Title of the Research Project

Studies on design of nano particulate formulations of propolis for anticancer activity

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Executive Summary of the Project

Propolis from apiculture is known for wide range of medicinal properties owing to its vast chemical constituents including polyphenols, flavonoids and anticancer agent Caffeic acid phenethyl ester (CAPE). In the present study extraction optimization of Indian propolis (IP) was done and standardized using a newly developed and validated High performance liquid chromatographic method for simultaneous estimation of Caffeic acid, Apigenin, Quercetin and CAPE. The standardised ethanolic extract of IP (EEIP) was screened for in-vitro cytotoxicity using sulforhodamine B (SRB) assay, in-vivo anti carcinogenic effect against Dalton's Lymphoma ascites (DLA) cells, hemolytic effect, acute toxicity and pesticide analysis. The EEIP was found to contain highest amount of total flavonoids (23.61+ 0.0452 mg equivalent of Quercetin/g) and total polyphenolics (34.82 + 0.0785 mg equivalent of Gallic acid/g) and all selected markers except Caffeic acid compared to all other extracts. EEIP showed better anticancer potential than CAPE on MCF-7 and HT-29 cell line and significant ($p<0.01$) in vivo anticarcinogenic effects against DLA in comparison with 5-fluorouracil. EEIP was found to be non hemolytic, free from pesticides and acute toxicity results revealed no hazardous signs or death of animals. EEIP exhibited better effects on inhibiting the tumor progression in vitro and in vivo, most likely because of synergistic activity of constituents present in the extract. From biosafety studies it can be concluded that the EEIP is safe for internal use and can be considered for development of suitable formulation.

Ethanolic extract of Indian propolis (EEIP) or caffeic acid phenethyl ester loaded Poly (ε-caprolactone) nanoparticles (denoted as EPL) to achieve improved solubility, controlled drug release and enhanced cytotoxic efficacy of EEIP as an intravenous drug delivery system. Formulation development, characterization and optimization were carried out by design of experiment approach. Developed formulations were evaluated in detail for nanoparticle

characterization and in terms of in vitro cytotoxicity study. Developed nanoparticles showed particle size, and encapsulation efficiency 190 ± 1 - 230 ± 2 nm, 62.66+ 1.20 - 67.18+ 1.80 % respectively. Optimized formulation EPL showed controlled drug release over a period of 48 h. Moreover, concentration of the drug needed for total growth inhibition of cells in a designed time period (TGI) was decreased by 33.06% for EPL as compared to EEIP in human breast cancer MCF-7 cells and 22.31% in human colon cancer cells HT-29 indicating improved cytotoxicity of EEIP.

Indian propolis (EEIP) or caffeic acid phenethyl ester loaded folic acid conjugated Poly (D,L-lactide-co-glycolide) nanoparticles (denoted as EPFA) were investigated to achieve improved solubility, controlled drug release, and synergized anticancer efficacy. Formulation development, characterization and optimization were carried out by design of experiment approach. Developed formulations were evaluated in detail for nanoparticle characterization and in terms of in vitro and in vivo cytotoxicity study. Developed EPFA showed the particle size, encapsulation efficiency 178 ± 5 - 205 ± 5nm, 73.16+ 1.89 - 76.37+ 1.89 respectively. Optimized formulation showed controlled drug release over a period of 54 h with no sign of blood toxicity. Moreover, concentration of the drug needed for growth inhibition of 50% of cells in a designed time period (GI50) was decreased by 43.34 %, for EPFA as compared to EEIP in human breast cancer MCF-7 cells indicating targeting with synergistic effect of EPFA. An improved anticancer effect was reflected in in-vivo Daltons Ascites Lymphoma model by reducing tumor cells count.

PROJECT FINAL REPORT:

Rationale and significance of the study:

Cancer is second largest non-communicable disease in world. Its prevalence in India is estimated to be around 2.5 million, with over 8, 00,000 new cases and 5, 50,000 deaths occurring each year due to this disease. More than 70% of the cases report for diagnostic and treatment services in the advanced stages of the disease, which has lead to a poor survival and high mortality rate along with immense emotional trauma and a major economical burden to patient's family. Hence there is need of development of advanced and cost effective treatment for various types of cancers as brain, breast, prostate, ovarian cancers.

Propolis, sometimes also called "bee glue", is a strongly adhesive, resinous substance that honeybees collect from various plants, transformed and used by bees to seal holes in their honeycombs, to smooth out the beehive's internal walls and to protect the entrance against intruders. Honeybees (*Apis mellifera* L.) collect the resin from cracks in plant barks and leaf buds. They masticate the resin and by doing so they add salivary enzymes to it. After this, they mix the partially digested material with beeswax and use it in their hive. Propolis is a mixture of various amounts of beeswax and resins collected by the honeybee from plants, particularly from flowers and leaf buds. Since it is difficult to observe bees on their foraging trips the exact sources of the resins are usually not known. Bees have been observed scraping the protective resins of flower and leaf buds with their mandibles and then carrying them to the hive like pollen pellets on their hind legs. It can be assumed that in the process of collecting and modeling the resins, they are mixed with some saliva and other secretions of the bees as well as with wax.

Caffeic acid phenethyl ester is one of the important compounds from propolis which has been investigated extensively for its anticancer activity. Several clinical trials demonstrating antimicrobial potential of propolis against highly resistant gram positive bacteria yeast, oral infections and chronic vaginal infection.

Caffeic acid phenethyl ester (CAPE) , a bioactive compound isolated from propolis has been known for a long time but first attracted attention in 1979, when a mixture of Caffeic acid esters, including phenethyl ester, was identified as the main antibacterial and antifungal principle of European propolis . Its popularity with researchers increased significantly after a publication reporting on the identification of CAPE as the main cytotoxic compound in propolis with preferential cytotoxicity on tumor cells. The authority of the senior author Nakanishi and the remarkable activity of the compound were the reason for the ever increasing interest of scientists in this compound in the years since 1988. At present, it is the most studied of all the individual constituents of propolis. In the Scopus scientific database, it was found that 474 hits for CAPE in title, abstract and/or keywords for life Sciences and Health Sciences, 183 of them being in Health Sciences. Different aspects of the biological and pharmacological activities of CAPE have been studied, including the mechanism of its action. The cytotoxicity of CAPE has also been reported. It was found to induce apoptosis of human pancreatic cancer cells and colon cancer cells. It inhibited the growth of C6 glioma

cells *in vitro* and *in vivo*. Other studies prove its chemopreventive effect CAPE has been proved to block the NF-κB activation process. Although the maintenance of appropriate levels of NF-κB activity is crucial for normal cellular proliferation, constitutive NF-κB activation is involved in the enhanced growth properties as seen in several cancers. Dietary intake of safe and nontoxic chemopreventives like CAPE May thus be beneficial for patients whose tumours express persistently high levels of activated NF-κB, such as non-small cell lung carcinoma, thyroid, colon, breast, stomach, squamous head and neck carcinomas.

Ethanol extracts of propolis have been found to transform human hepatic and uterine carcinoma cells *in vitro*, and to inhibit their growth. Propolis was also found to have a cytotoxic and cytostatic effect *in vitro* against hamster ovary cancer cells and sarcoma-type tumors' in mice. A substance called Artepillin C has been isolated from propolis, and has been shown to have a cytotoxic effect on human gastric carcinoma cells, human lung cancer cells and mouse colon carcinoma cells in vitro.

Propolis is widely used in Indian folk medicine for the treatment of stomach ulcers. The preventive and curative effects of Indian propolis (propolis samples from Mudivaithanendal, Tamil Nadu) for ulcers were evaluated using models of acute gastric lesions induced by ethanol and indomethacin in rats. Moreover, the effects of ethanolic extract of propolis on gastric content volume, total acidity and pH, using the pylorus ligated model were also evaluated. Animals pretreated with propolis extract showed a significant reduction in lesion index in both ethanol and indomethacin induced ulcer models in a dose dependent manner when compared to the control group. Similarly, post-treatment with propolis (300 mg/kg body weight) for a period of 15 days revealed a statistically significant improvement in the ulcer healing process $p < 0.05$. In the pylorus ligated model, it was observed that the Indian propolis extract displayed an antisecretory activity, which led to a significant reduction in the gastric juice volume, total acidity and pH. These findings indicated that, Indian propolis displays both ulcer preventive and ulcer curative properties and provides a scientific rationale for the use of propolis in the traditional medicinal system.

A survey of work one in the research area and the need for more research:

Antitumor activity of ethanol extract of propolis has been studied on the percentage of TUNEL positive human breast cancer cell line MCF-7. The study showed that apoptosis induction is strongly dependent on th concentration and dilutions of EEP. In the antitumor activity, dilutions of EEP 0.125 and 0.063 mg/ml were more effective (17.5–100%) than dilutions of EEP 0.25 and 0.5 mg/ml (5.11–18.97%) The over activity of caspase-6 induced by EEP is stronger than the induced activity of caspase-8 and -9 in MCF-7 cells, which confirms the involvement of intrinsic caspase pathway of apoptosis and the antitumor activity of propolis.

Indian stingless bee propolis has a complex chemical nature and is reported to possess various medicinal properties. In the present study, anticancer activity of the ethanolic extract of propolis was explored by testing the cytotoxic and apoptotic effect in four different cancer cell lines, namely, MCF-7 (human breast cancer), HT-29 (human colon adenocarcinoma),

Caco-2 (human epithelial colorectal adenocarcinoma), and B16F1 (murine melanoma), at different concentrations. Cytotoxicity was evaluated by MTT assay and Trypan blue dye exclusion assay. EEP at a concentration of 250 μg/ml exhibited ≥50% mortality in all cell lines tested (i.e., IC50 value). EEP revealed a concentration and time dependent cytotoxic effect. Apoptosis was estimated by differential staining (ethidium bromide/acridine orange) and TUNEL (deoxynucleotidyl transferase-dUTP nick end labeling) assay. Light microscopy and atomic force microscopy demonstrated morphological features of apoptosis in all the cell lines after treatment with 250 μg/ml EEP for 24 h. Thus, early onset of apoptosis is the reason for anticancer activity of Indian stingless bee propolis. Further, the antioxidant potential of Indian stingless bee propolis was demonstrated to substantiate its anticancer activity. [5]

Seven different propolis extracts from Turkey were investigated on the human breast cell line MCF-7, and it was concluded that propolis may exert antitumor effects by increasing apoptosis through the caspase pathway. Although propolis may exert a direct effect on different tumor cells in vitro, the administration of propolis to animals or humans is followed by its solubility and systemic bioavailability. Some isolated compounds have also been investigated and could be responsible for the antitumor action of propolis. However, since the composition of propolis is very complex, more compounds should be investigated in tumor assays both in vitro and in vivo, as well as the synergistic effects between them. The main mechanism of action of propolis involves apoptosis, cell cycle arrest and interference in metabolic pathways. Apoptosis, a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage, can provide novel potential drug targets that are able to induce death in cancer cells.

CAPE is effective in many models of human cancer, including BC as we have previously shown. CAPE affects genes associated with tumor cell growth and survival, angiogenesis and chemoresistance. We demonstrate that these are related in part to CAPE's role as a histone deacetylase inhibitor, a class of drugs designated as epigenetic agents that modulate the activities of oncogenes and tumor suppressor genes. CAPE and propolis, cause an accumulation of acetylated histone proteins in MCF-7 (ER+) and MDAMB- 231 (ER−/PR−/Her2-) cells with associated decreases in ER and PR in MCF-7 cells, and upregulation of ER and decrease in EGFR in MDA-231 cells. [8]

The major problem with propolis reported is its poor water solubility which hampers its nutritional and therapeutic effectiveness. Few attempts have been reported to improve water solubility and availability of propolis, US patent 5,922,324-1999 reports propolis extract with improved water-solubility using hydro alcoholic solution.

The cytotoxicity of propolis nanoparticles against MCF-7, A375, PC3 and PANC-1 cancer cell lines were tested using MTT assay and the minimal concentration toxic to the cancer cells were found. The cell uptake studies of propolis nanoparticles on MCF-7 cells demonstrated internalization of the nanoparticles by the cancer cells. All these studies revealed that propolis nanoparticles could be a good substitute for the existing materials against cancer.

The presence of the bioactive compounds with phenolic OH groups, which are susceptible to oxidation in the propolis extracts (especially flavonoids and cinnamic acid derivatives) confer to the extracts significative antioxidant activity (determined by DPPH method), as well as for the propolis extracts/β-cyclodextrin micro/nanoparticles. Furthermore, it can be observed a retard effect in the case of complexes, therefore a longer antioxidative activity, due to the slower. The efficiency of the ethanol and water extracts of Indian propolis towards Ag and Au nanoparticles synthesis was compared with that of naturally occurring hydroxyflavonoids, pinocembrin and galangin isolated from Indian propolis; which are equally efficient in the rapid synthesis of Ag and Au nanoparticles and stabilization of the resultant particles.

Polymeric nanoparticle encapsulated formulation of propolis (propolis nanofood) utilizing micellar aggregates of cross linked and random copolymers have been prepared to enable parenteral administration of propolis in an aqueous phase to explore anticancer potential in clinical area.

CAPE and some other constituents of propolis which are responsible for anticancer activity having wide range of anticancer therapeutic efficacy but they possess low aqueous solubility and owing to high lipophilicity they possesses very low oral bioavailability, being lipophilic in nature, it will be taken up by RES system, rendering it less bioavailable at non-RES sites such as breast and brain. It is the major drawback in their use as a potential drug for cancer treatment. Also due to concentration dependant and time dependant anticancer activity of ethanolic extract of Indian propolis it is necessary to design suitable extraction pretreatment and formulation development approach for Indian propolis extract and CAPE .So it is necessary to formulate suitable targeted drug delivery system for propolis and also to characterize and evaluate *in-vivo, in -vitro* for anticancer activity

Brief objective of the project:

The objectives of the project:

1. To establish analytical methodology for standardization of Indian propolis of selected origin.
2. To design and develop targeted nanoparticulate formulations of Indian propolis for cancer therapy.
3. To formulate ligand mediated therapeutically effective formulation of Indian propolis for anticancer activity.
4. To design and develop targeted nanoparticulate formulations of caffeic acid phenethyl ester for cancer therapy.
5. To formulate ligand mediated therapeutically effective formulation of caffeic acid phenethyl ester for anticancer activity.

Plan of Study

Methodology

- Preliminary study: Color, Light microscopy, Scanning Electron Microscopy.
- Development of quality control analytical method for raw Indian Propolis: Total polyphenol content: The content of total polyphenols was quantified according to the Folin-Ciocalteau spectrophotometric method using gallic acid as reference standard (Singleton, Joseph & Rossi, 1965). Further propolis was standardized with respect to CAPE content.
- Development and optimization of method of extraction for Indian Propolis
- Analysis of the prepared propolis extract: Total polyphenol content, , CAPE content, etc. Safety and allergenicity of the extract.
- Nanoparticulate Formulation development using prepared propolis extract:

  Preparation and optimization of polymeric nanoparticles using ligands

  - Characterization of optimized nanoparticulate system for:

  Physicochemical characterization tools as

  SEM, DSC, XRD, NMR, FTIR, TEM, AFM, HPLC, GC, and LCMS.

  - Biological characterization

  In vitro cell culture study for anticancer activity

  In vivo toxicity and biodistribution study in laboratory animals

PART 1

Part 1

Standardization, Chemical Profiling, *In Vitro* Cytotoxic Effects on MCF 7 and HT 29 Cell Lines, *In Vivo* Anti Carcinogenic Potential and Biosafety Profile of Indian Propolis

Introduction

Natural products have proven as rich source of various constituents that have found to possess anti tumor activity and applications in cancer chemotherapy [1]. Propolis is an important healthy food ingredient which has nutritional and medicinal properties obtained from apiculture [2, 3]. Propolis has been widely used for its nutritional and medicinal values since ancient time in Greece, Roman empire, Egypt and various countries. Even today it continues as popular remedy as natural products and as a healthy food [4]. It is a resinous material collected by honey bees specifically *Apis mellifera* from the plant parts, exudates and buds is known for its nutritional and medicinal properties obtained from apiculture [2]. Apart from use of propolis as a nutritional and healthy food constituent, its use in human health in folk medicine [5] has been reported from ancient time. Propolis of various regions is known to exhibit various activities including antifungal, antibacterial and anticancer activity [6, 7, 8]. Various *in vitro* and *in vivo* studies on propolis and its isolated constituents [9] have been reported for anticancer activity. The anticancer activity of propolis is attributed to Caffeic acid phenethyl ester (CAPE) in addition to poly phenols and flavonoids [10, 11] CAPE is a specific inhibitor of NF-κB. In low concentrations it significantly suppresses the lipoxygenase pathway of arachidonic acid metabolism during inflammation. It completely prevents the production of reactive oxygen species (ROS) in human neutrophils [11] and the xanthine/xanthine oxidase system at a concentration of 10 μM.

Although propolis is extensively studied globally, the research on Indian propolis is at infancy. There is lack of studies on extraction optimization, analytical method and biosafety study on Indian propolis except few [12-17]. So in present study attempt has been made for extraction of Indian propolis by suitable method, standardize with respect to selected markers by new analytical method, evaluate acute toxicity, biosafety, *in vitro and in vivo* anticancer activity, which are pre requirement to develop formulation of Indian propolis to be used as drug delivery system.

Experimental

Material

The Indian propolis sample collected in month of December was purchased from local bee keeper from the Bharatpur region of Rajasthan, India and authenticated by Central Bee Research and Training Institute (CBRTI) Pune. Apigenin (>99% purity) were purchased from Natural remedies India private limited, Bangalore, India. Caffeic acid, Caffeic acid phenethyl ester (>99% purity) were purchased from Sigma Aldrich, Bangalore, India. All reagents were used as of analytical grade from Merck, India.

Instruments

Moisture content was analyzed using halogen moisture analyzer. UV-vis spectrophotometer and HPLC system were from Jasco Corporation, Tokyo, Japan; HPLC comprises of Jasco AS 1555 auto sampler with 10 µL loop capacity per injection, Borwin version software, pump PU 1580 and detector UV 1575. COBAS MIRA PLUS-S Auto analyzer, Roche Switzerland, COBAS MICROS OT 18 from Roche were used for biochemical and hematological studies respectively. Hi-Tech instruments MAX MAT auto analyzer was used blood analysis.

Animals

Healthy female mice (20–25 g) supplied by National Institute of Biosciences Pune, India were selected for acute toxicity study. The studies were performed according to Organization for Economic Cooperation and Development (OECD 425) guidelines (18). The study protocol was approved by the Institutional Animal Ethics Committee (protocol number: CPCSEA/QA/06/2016).

Total balsam content

Accurately weighed 1g of crude propolis was dissolved in 10 mL of ethanol, filtered and filtrate was evaporated to dryness until constant weight was obtained, and the ethanol soluble fraction was taken as percentage of balsam in the crude propolis sample (19).

Extraction and characterization of Indian propolis

Extraction

10g of crude propolis was extracted with 30 mL of hexane by sonication for 30 min to remove the wax and filtered to obtain Hexane extract of Indian propolis (HEIP). The mark was further extracted with ethyl acetate by sonication method and ethanol by soxhlet method at 60^0C to obtain ethyl acetate extract of Indian propolis (EAEIP) and Ethanolic extract of Indian propolis (EEIP) respectively. Further, mark was extracted with water by soxhlet extraction at 100^0C to obtain water extract of Indian propolis (WEIP). All extracts were stored in 2-8^0 C and used for further evaluation (12).

Total flavonoids and total polyphenol contents

Total flavonoids and polyphenol contents were determined by following methods reported by Marinova et al., 2005 and results were expressed in the form of mg equivalence of Quercetin/g for flavonoids and mg equivalence of Gallic acid/g for polyphenols (20, 21, 22).

Method development and validation

Individual stock solutions of Caffeic acid, Apigenin, Quercetin and CAPE were prepared in ethanol to obtain 1 mg/ mL solutions, diluted suitably to obtain working standards and stored in refrigerator (4^0 C).

Accurately weighed 30 mg of extracts were dissolved in 10 mL of ethanol and diluted suitably to achieve concentration of 3000 µg/mL solution.

HPLC separation was optimized using the aliquots of standard solutions ranging from 20 to 70 µg/mL for Apigenin, Caffeic acid and CAPE and 30 to 80 µg/mL for Quercetin and analyzed. The proposed method was validated as per ICH guidelines (ICH Q2 (R1) 2005). Accuracy is expressed as % recovery by adding of known amount of analyte in the sample. It was established across the specified range of analytical procedure. It was determined by calculating recovery of Caffeic acid, Apigenin, Quercetin and CAPE by standard spiking method. For determination of intraday and interday precision, solutions of 3 different

concentrations were analyzed at 3 different time intervals in same day and different days and percent RSD was calculated.

Robustness of the method determined by measuring the effect of small and deliberate changes in the analytical parameter on the retention time and peak area. The parameters selected were mobile phase concentration, flow rate and wavelength. While one parameter was altered remaining were kept constant. Standard deviation and percent standard deviation of peak area was calculated.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on standard deviation of the response and the slope as per ICH guidelines

All extracts were standardized for Caffeic acid, Apigenin, Quercetin and CAPE using developed HPLC method.

Total moisture content and pesticide analysis

Total moisture content of EEIP was determined using Mettle Toledo HB 43 Moisture analyzer. 3 g of raw propolis kept on pan at 100^0C until constant weight was obtained.

Total pesticide content of EEIP was analyzed by Marco et al., 2012 using 410 Proster binary LC with 500 MS IT PDA detectors and EEIP was analyzed to check various types of pesticides (23).

In vitro anticancer study

In-vitro anticancer study was carried out at advanced centre for treatment, research and education in cancer (ACTREC, Navi Mumbai). The study was carried out by *in vitro* Sulforhodamine B assay method. The cell lines of MCF-7 (Human breast cancer) and HT-9 (Colon cancer) were procured from NCCS, Pune. The cytotoxicity study protocol for *in vitro* Sulforhodamine B assay was followed by method described by Bothiraja et al., 2013 (24).

Hemolysis and plasma protein binding study

Hemolytic effect and the plasma protein binding ability of EEIP was evaluated using method described by Bothiraja et al., 2013 (24).

Acute toxicity study of EEIP

The acute toxicity study of EEIP was carried out as per OECD guidelines (OECD Test No. 425, 2008). The mice were randomly divided into six groups (n= 6/sex). The first group (control group) received distilled water orally. Groups 2–6 were orally treated with EEIP (dispersed in 0.5%w/v carboxymethylcellulose) with doses 55, 175, 550, 1750, and 2000 mg/kg of body weight, respectively. General behavioral changes, sign of toxicity, and mortality were observed for 1 hr after treatment and intermittently for 4 hrs and thereafter over a period of 24 hrs. Mice were further observed for up to 14 days for behavioral change and sign of toxicity (18).

***In vivo* anticancer study**

Selection Grouping and Acclimatization of Laboratory Animal

Male Swiss albino mice (20-25 gm) were used for the study. They were acclimatized to laboratory environment at (temp 25±2°C) and 12 hrs dark /light cycle with diet and water.

Induction of cancer using DLA cells

Dalton's Lymphoma ascites (DLA) cells were supplied by Amala cancer research center, Trissur, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were

aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilution were made so that total cell should be 1 x 10^6. Tumor growth in the mice was allowed for minimum seven days before starting treatments.

Treatment Protocol

Animals were divided in to six group of six each. G2- G6 were injected with DLA cells (1 x 10^6 cells per mouse) intraperitoneally. Group 1(G1) served as the normal control, Group 2 (G2) served as the tumor control. Group 1 and 2 receives normal diet and Water. Group 3 (G3) served as the positive control and was treated with injection 5-fluorouracil at 20 mg/kg body weight, intraperitoneally. Group 4 (G4), Group 5 (G5) and Group 6 (G6) were treated with EEIP at a dose of 100,200 and 400 mg/kg intraperitoneally.

Evaluation of anticancer activity

Drug treatment was given after 24 hrs of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital plexus bleeding and the following parameters were evaluated. Hematological parameters like WBC, RBC count , Hb content, platelet count and packed cell volume; Serum enzyme and lipid profile which include total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP); and derived parameter like body weight, life span (%) and cancer cell count were evaluated. The results are expressed as mean ± S.E.M. The evaluation of the data was done using one way ANOVA followed by Newman-Keul's multiple comparison test; $p< 0.01$ implied significance (25, 26, 27, 28).

Result and discussion

Preliminary analysis revealed that crude Indian propolis was yellowish brown in color, and sticky. It was having a typical odor with a bitter taste. The pollens of *Brassica campestris, Eucalyptus species, Cocos nucifera, Punica grantanum* and few grains belonging to *Asterace* family was present in purchased Indian stingless bee propolis. Total balsam content was found to be 46% w/w. The total extraction yield of each extract was 18.23% w/w, 7.11% w/w, 49.00% w/w and 10.40% w/w for Hexane extract of Indian propolis (HEIP), ethyl acetate extract of Indian propolis (EAEIP), ethanolic extract of Indian propolis (EEIP) and water extract of Indian propolis (WEIP) respectively. Total polyphenols contents were found to be 18.06 $\pm$ 0.064 and 34.82 $\pm$ 0.078 mg equivalent of Gallic acid/g in EAEIP and EEIP respectively. Flavonoids content of EEIP and EAEIP was found to be 23.61 $\pm$ 0.045 and 11.30 $\pm$ 0.011 mg equivalent of Quercetin/g respectively. The results shows significant amount of polyphenol and flavonoids are present in EAEIP and EEIP sample.

A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 mL/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 µ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 µL, and the detection wavelength was set at 331 nm. Temperature was maintained at $25^0 \pm 2^0$ C.

Acceptable response and detection of four markers obtained at wavelength 331 nm. Each run was followed by 10 min wash with methanol. Calibration curves were plotted which were found to (n=3 repetitions of each point) be linear in the range of 20-70 µg/mL for Caffeic

acid, Apigenin and CAPE, 30-80 µg/mL for Quercetin and with good correlation coefficient. The linear regression data, LOD and LOQ for all four markers are shown in Table 1. Average retention time ± standard deviation were observed and results are shown in Table 2. Representative chromatogram is shown in Fig. 1.

The intra-day and inter-day precision as % RSD for all four markers are shown in Table 3. Satisfactory recoveries for all four markers were obtained as shown in Table 4. The robustness result showed that the peak areas remain unaffected (% RSD < 2), indicates the proposed method is robust Table 5. The system suitability was performed by calculating chromatographic parameters namely symmetry factor, and theoretical plates. Values obtained were shown in Table 2.

Table 1. Linear regression data

Parameters	Caffeic acid	Quercetin	Apigenin	CAPE
Linearity range (µg/mL)	20-70	30-80	20-70	20-70
Regression equation	y = 5024.x + 1464	y = 37619.x – 79941	y = 70037.x – 1377000	y = 56240.x – 60582
r^2	0.998	0.999	0.999	0.998
Slope	5024	37619	70037	56240
Intercept	1464	79941	1377000	60582
LOD (µg/mL)	2.413	1.649	1.752	2.499
LOQ (µg/mL)	7.312	4.997	5.309	7.572

r^2- square of correlation coefficient, LOD-Limit of detection, LOQ- Limit of quantitation, µg/mL – microgram per milliliter.

Table 2. System suitability parameters of chromatogram for Caffeic acid, Quercetin, Apigenin and Caffeic acid phenethyl ester

Parameters	Proposed RP-HPLC method							
	Caffeic acid	% RSD	Quercetin	% RSD	Apigenin	% RSD	CAPE	% RSD
Retention time (min)	1.993	0.125	3.153	0.073	3.757	0.138	5.804	0.069
Peak symmetry	1	-	1	-	1	-	1	-
Theoretical plates	2795	0.076	3496	0.028	5202.8	0.898	7359.5	0.155

% RSD- percent relative standard deviation

Table 3. Intra and inter day precision of the HPLC method (n=3)

Marker compounds	Concentration µg/mL	Intra/inter day concentration obtained (µg/mL)		Precision obtained % RSD	
		Intraday	Interday	Intraday	Interday
Caffeic acid	20	19.600	21.050	0.963	0.230
	30	29.830	30.230	0.781	1.520
	40	40.180	40.530	1.662	0.628
Quercetin	30	31.540	31.820	0.483	0.286
	40	40.340	41.220	0.011	0.265
	50	51.820	50.480	0.010	0.116
Apigenin	20	20.803	20.800	0.001	0.001
	30	30.914	30.910	0.565	0.550
	40	41.251	41.250	0.709	0.665
CAPE	40	41.240	41.720	1.870	1.369
	50	49.750	50.140	0.126	1.147
	60	59.210	59.170	0.125	0.619

n- Number of replicates, % RSD- percent relative standard deviation, µg/mL – microgram per milliliter.

Table 4
Result of recovery studies (n=3)

Parameters	Amount taken µg/mL	Amount added µg/mL	Total Amount µg/mL	Amount found ± SD µg/mL	% Recovery ± % RSD
Caffeic acid	20	16	36	35.070±0.398	97.410±1.100
	20	20	40	39.800±0.247	99.520±0.620
	20	24	44	43.500±0.064	98.940±0.148
Quercetin	30	27	57	56.990±0.074	99.820±0.128
	30	30	60	59.650±0.075	99.410±0.125
	30	33	63	62.320±0.081	98.920±0.129
Apigenin	20	16	36	35.190±0.334	97.750±0.924
	20	20	40	39.760±0.277	99.330±0.697

	20	24	44	43.460±0.563	98.770±1.260
CAPE	20	16	36	35.900±0.137	99.720±0.384
	20	20	40	39.340± 0.135	98.350±0.336
	20	24	44	43.790 ±0.053	99.530±0.125

n-no of replicates, SD- Standard deviation, % RSD- percent relative standard deviation, µg/mL – microgram per milliliter.

Table 5. Robustness study (n=3, 20 µg/mL for Caffeic acid, Apigenin, 30 µg/mL for Quercetin and 40 µg/mL for CAPE)

Parameters	**SD of Area (n=3)**				**% RSD for Area (n=3)**			
	Caffeic acid	**Quercetin**	**Apigenin**	**CAPE**	**Caffeic acid**	**Quercetin**	**Apigenin**	**CAPE**
Mobile phase composition Methanol ± 1 Ml	42.140	6705.858	458.210	10237.890	0.038	0.601	0.848	0.608
	383.310	6711.350	608.300	12365.350	0.166	0.602	1.120	0.720
Elution flow rate ± 0.1 mL	136.930	6705.850	501.170	18224.900	0.124	0.602	0.930	1.070
	42.140	6481.110	749.940	21039.990	0.038	0.580	1.350	1.240
Detection wavelength ± 2 nm	47.040	16552.860	76.440	42.770	0.041	1.470	0.141	0.002
	42.140	6976.690	194.160	17883.450	0.038	0.620	0.366	1.066

n-no of replicates, SD- Standard deviation, % RSD- percent relative standard deviation, nm- nanometer.

The percent amount of Apigenin, Quercetin and CAPE in EEIP was found to be 1.005 ± 0.070, 1.344 ± 0.021 and 0.677 ± 0.002 respectively. Amount of Caffeic acid in WEIP was found to be 1.019 % ± 0.016. Representative chromatograms are shown in Fig. 2.

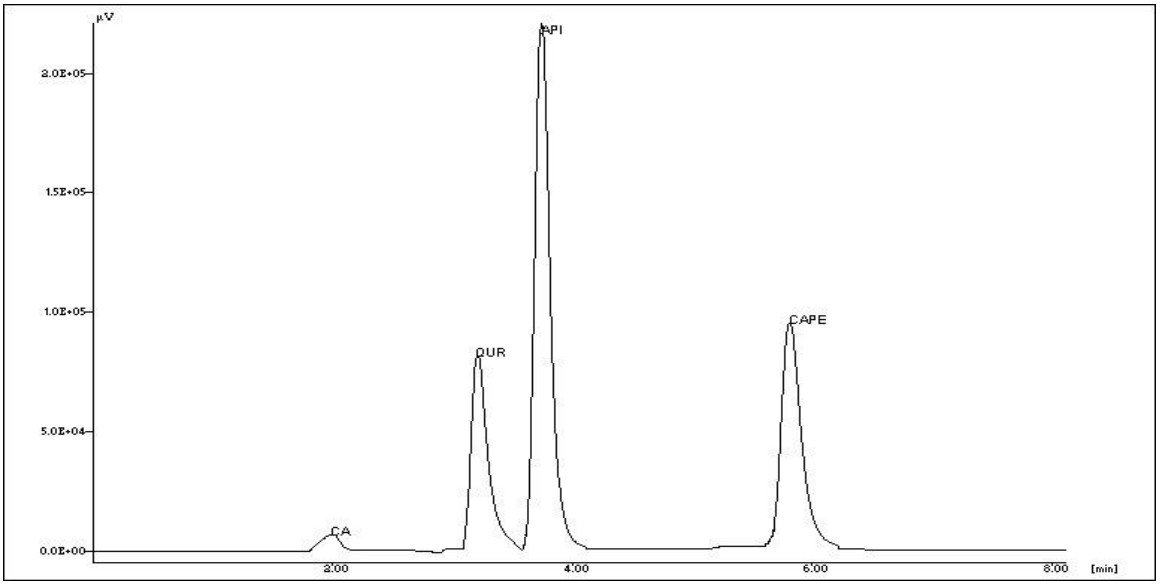

Fig. 1 RP-HPLC Chromatogram of Caffeic acid (CA), Quercetin (QUR), Apigenin (API) and Caffeic acid phenethyl ester (CAPE).

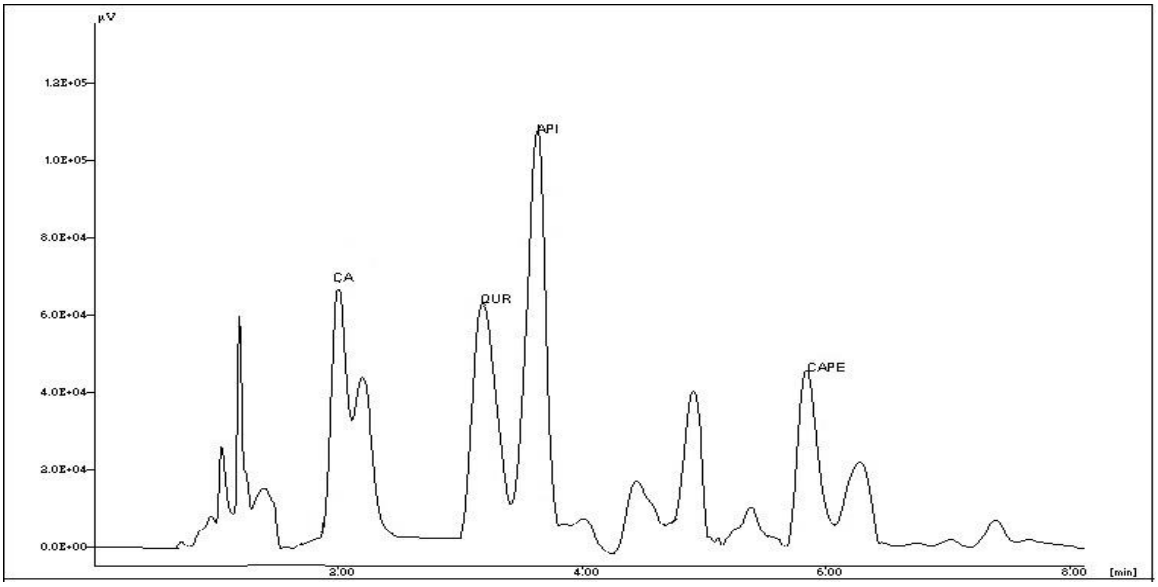

Fig. 2 RP-HPLC Chromatogram of Ethanolic extract of Indian propolis (EEIP) showing presence of Caffeic acid (CA), Quercetin (QUR), Apigenin (API) and Caffeic acid phenethyl ester (CAPE).

The new HPLC method was and developed and validated for simultaneous estimation of markers, from the linear regression data it was found that the developed method is linear and sensitive. Baseline did not show any significant noise and there were no other interfering peaks around the retention time of Caffeic acid, Apigenin, Quercetin and CAPE, indicating proposed RP-HPLC method is specific. The relative standard deviation values of the intraday and inter day precision study are within limit as per ICH guideline and method showed good precision.

The proposed RP-HPLC method was found to be reliable for simultaneous quantification of selected markers and validation parameters are in the limits of ICH guidelines.

Results showed that the EEIP contains presence of Apigenin, Quercetin and Caffeic acid phenethyl ester whereas WEIP showed the presence of Caffeic acid. Absences of markers were observed in hexane and ethyl acetate extracts.

Total moisture content in crude propolis was 3.5% w/w. About 113 pesticides were tested including phorate, ediphenphos, dimethoate and tricyclazole. No significant amount of pesticides was observed in EEIP indicate safety of propolis for internal use.

In vitro anticancer activity of EEIP was investigated and compared with the pure CAPE against human breast cancer MCF-7 and colon cancer HT-29 cells using *in vitro* SRB assay. The result illustrated in Table 6 indicates that EEIP shown better activity than pure CAPE. The total growth inhibition (TGI) concentration value of EEIP was found to be (31.10 µg/mL) and (39.90 µg/mL) whereas Caffeic acid phenethyl ester was 46.00 µg/mL and 47.20 µg/mL on MCF -7 breast cancer cells and HT-29 colon cancer cell lines respectively. Fig. 3 (A-F) is the representative microscopic images obtained from *in vitro* anticancer studies on breast cancer cell line MCF-7 and colon cancer cell line HT-29 respectively. Results and microscopic images (Fig. 3 (A-F)) reveal that the EEIP exhibited more potent activity on both cancer cell lines than that of the pure CAPE.

Table 6 . TGI and GI_{50} values of ADR, CAPE and EEIP on HT-29 colon cancer cell line and MCF-7 breast cancer cell line.

Samples	HT-29 cell line TGI (µg/mL)	HT-29 cell line GI_{50} (µg/mL)	MCF-7 cell line TGI (µg/mL)	MCF-7 cell line GI_{50} (µg/mL)
ADR	< 10	< 10	<10	<10
CAPE	47.20	20.10	46.00	<10
EEIP	39.90	16.50	31.10	<10

Values are presented as mean (n = 3).

EEIP- Ethanolic extract of Indian Propolis; CAPE- Caffeic acid phenethyl ester; ADR- Adriamycin positive control, TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produce 50% inhibition of cells, MCF 7- cell line, HT-29- Human colon cancer cell line, µg/mL – microgram per milliliter.

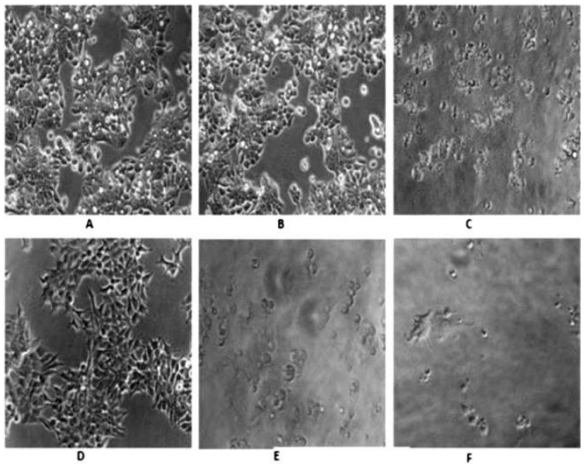

Fig. 3 *In vitro* cytotoxicity study on HT-29 Cell line. A) Positive control. B) CAPE Treated C) EEIP Treated and *in vitro* cytotoxicity study on MCF-7 Cell lines. D) Positive control. E) CAPE Treated F) EEIP Treated.

In vitro anticancer activity of EEIP was investigated and compared with the pure CAPE against human breast cancer MCF-7 and colon cancer HT-29 cells using *in vitro* Sulforhodamine B (SRB) assay. SRB assay is a well known and sensitive method for evaluating cytotoxic activity against both cancer and noncancerous cell lines. It is advantageous over other contemporary cytotoxicity assays; it is independent of cell metabolic activity and also not interfered by test compounds.

The GI 50 and TGI of EEIP were determined for two cancer cell lines by SRB assay. EEIP was observed to be cytotoxic in dose dependent manner on all two cancer cell lines. The cytotoxic ability of crude extracts can be attributed to their phytochemical constituents. The results obtained from GI 50, reveal that both CAPE and EEIP are comparable to adriamycin and can be considered to have anticancer potential. The results showed that EEIP possess comparatively better anticancer potential on MCF-7 breast cancer cell line than HT-29 human colon cancer cell line. Also I both cell line EEIP have better anticancer potential than CAPE that may be because of synergistic activity of other polyphenols and flavonoids present in EEIP.

It has been postulated that polyphenols and flavonoids possess anticancer activity by several mechanisms including decrease of ROS, modulation of signaling pathways and down regulation of nuclear transcription factor kappa B (NF-κB). For MCF-7 cell line, the reason for the better growth inhibition might be due to synergistic effect of various polyphenols and flavonoids present in EEIP. For *in vivo* application it is necessary to evaluate the biosafety study that is hemolysis and protein binding assay.

The hemolytic potential of the EEIP was evaluated using optical density method. The result indicates that the hemolysis rate for 0.25, 0.5, 0.75, 1.0 and 1.5 mg/ml concentration of EEIP were 1.5, 2, 2.8, 3.4 and 4.1 % respectively. The acceptable hemolysis rate (less than 3%) [24] Shown by EEIP denotes its non hemolytic property up to 750 µg/mL. EEIP showed no or less effect on red blood cells. So the EEIP may be considered as biosafe for internal use.

The plasma protein binding rate for EEIP was obtained was 57.34 ± 1.36 %. The plasma protein binding rate of EEIP was found slightly higher range indicates the need for development of suitable formulation to use EEIP internally as drug delivery system. Oral administration of EEIP in acute dose up to 5 g/kg was found to be safe.

No death or hazardous sign on tested animals were recorded during first 24 h as well as 14 days of observation after oral treatment of EEIP at the doses of up to 2000 mg/kg, respectively.

In the DLA tumor control group, the average life span of animal was found to be 48% where as EEIP at a dose of 100,200,400 mg/kg body weight increase the life span (Table 7) these values were significant *($p<0.01$).* However the average life span of 5-FU treatment was found to be 94%, indicating its potent antitumor nature. The antitumor nature of EEIP at a dose of 100,200,400 mg/kg body weight was evidenced by the significant reduction *($p<0.01$)* in percent increase in body weight of animal treated when compared to DLA tumor bearing

mice. It was also supported by the significant reduction *(p<0.01)* in packed cell volume and viable Tumor cell count when EEIP at a dose of 100,200,400 mg/kg body weight treatments compared to the DLA tumor control.

As shown in (Table 8) RBC, Hb, Platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with EEIP at a dose of 100,200,400 mg/kg body weight significantly increases *(p<0.01)* the Hb content, RBC, Platelets and significantly decreased *(p<0.01)* the WBC count to about normal level.

The inoculation of DLA cells caused significant *(p<0.01)* increase in the level of total cholesterol, aspartate amino transferase, alanine amino transferase, alkaline phosphatase in the tumor control animals(G_2), when compared to the normal group. The treatment with EEIP at the dose of 100,200 and 400 mg/kg body weight significantly *(p<0.01)* reversed these changes towards the normal level (Table 9). The treatment with standard 5-FU also gave similar results.

Table 7

Effect of EEIP on the life span, body weight and cancer cell count of tumor induced mice.

Treatment groups	Number of animals	Percent Increase in Life span	Increase in Body weight (grams)	Cancer cell count mL X 10^6
G_1	6	>>30 days	02.22±0.68	-
G_2	6	48%	$9.44±1.86^{a**}$	$2.75±0.80^{a**}$
G_3	6	96%	$5.66±0.42^{b**}$	$1.30±0.22^{b**}$
G_4	6	88%	$5.45±0.32^{b**}$	$1.42±0.30^{b**}$
G_5	6	89%	$5.36±0.30^{b**}$	$1.40±0.28^{b**}$
G_6	6	90%	$5.30±0.28^{b**}$	$1.38±0.24^{b**}$

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6– Treatment control EEIP 100,200,400 mg/kg respectively.

All values are expressed as mean ± SEM for 6 animals in each group.

One-way ANOVA followed by Newman-Keul's multiple comparison test.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table 8 . Effect of EEIP on Hematological parameters

Treatment groups	Total WBC Cells /mL x 10^3	RBC Count Millions /mm^3	Hb gm/Dl	Packed Cell Volume (Percent)	Platelets Lakhs/mm^3
G_1	10.35 ±1.05	4.55±1.95	12.90 ±1.95	14.25±2.44	3.60±0.95
G_2	15.30 ±2.60^{a**}	2.70±0.98^{a**}	6.80 ±0.95^{a**}	38.36±3.35^{a**}	1.70±0.42^{a**}
G_3	12.30 ±1.34^{b**}	4.05±1.62^{b**}	11.90±1.48^{b**}	16.40±1.40^{b**}	2.94±0.50^{b**}
G_4	12.12±1.26^{b**}	4.06±1.50^{b**}	12.22±1.52^{b**}	17.30±2.36^{b**}	3.30±0.65^{b**}
G_5	12.05±1.22^{b**}	4.08±1.60^{b**}	12.25±1.55^{b**}	17.24±2.30^{b**}	3.36±0.68^{b**}
G_6	11.85±1.18^{b**}	4.12±1.65^{b**}	12.35±1.60^{b**}	17.20±2.26^{b**}	3.40±0.70^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6– Treatment control EEIP 100,200,400 mg/kg respectively.

All values are expressed as mean ± SEM for 6 animals in each group.

One-way ANOVA followed by Newman-Keul's multiple comparison test.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table 9 . Effect of EEIP on serum Enzymes and lipid proteins

Treatment groups	Cholesterol (mg/dL)	TGL (mg /dL)	AST (U/L)	*ALT (U/L)*	ALP (U/L)
G_1	108.85±3.05	136.85±2.55	36.40 ±1.65	31.28 ±1.45	132.28 ±2.08
G_2	146.95±4.34^{a**}	220.28±4.40^{a**}	78.6±2.94^{a**}	62.32±2.60^{a**}	265.30±4.35^{a**}
G_3	126.30±3.84^{b**}	169.15±2.65^{b**}	44.40 ±1.72^{b**}	34.52±1.70^{b**}	154.45±2.40^{b**}
G_4	117.26±3.42^{b**}	160.08±2.55^{b**}	42.44 ±2.30^{b**}	35.28±1.55^{b**}	162.45±2.22^{b**}
G_5	115.18±3.38^{b**}	156.25±2.50^{b**}	41.60 ±2.20^{b**}	34.90±1.42^{b**}	160.48±2.18^{b**}
G_6	113.36±3.26^{b**}	153.30±2.46^{b**}	40.90 ±2.16^{b**}	34.80±1.38^{b**}	158.45±2.15^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6 – Treatment control EEIP 100,200,400 mg/kg respectively, Total Cholesterol (TC), Triglycerides (TGL),

Aspartate amino Transferase (AST), Alanine amino Transferase (ALT), Alkaline Phosphatase (ALP), U/L- units per liter

All values are expressed as mean ± SEM for 6 animals in each group.

One-way ANOVA followed by Newman-Keul's multiple comparison test.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

The antitumor nature of EEIP was evidenced from the significant *(p<0.01)* increase of average life span, reduction in percent increase in body weight, reduction in packed cell volume and viable tumor cell count compared to the DLA tumor control. RBC count, Hb, Platelets were decreased and WBC count was significantly *(p<0.01)* increased in the DLA control group compared to the normal control group. Treatment with EEIP at the dose of 100,200 and 400 mg/kg, significantly *(p<0.01)* increased the Hb content, RBC, Platelets and decreased the WBC count. However, the standard 5FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

In DLA tumor bearing, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. Treatment with EEIP at the dose of 100,200 and 400 mg/kg inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the lifespan of animals. It may be concluded that EEIP at the dose of 100, 200 and 400 mg/kg increases the life span of DLA bearing mice by decreasing the nutritional fluid volume and arresting the tumor growth thus exhibiting antitumor activity against DLA bearing mice.

Myelo suppression and anemia are the common problems in cancer chemotherapy. Reduction in hemoglobin content results in anemia in tumor bearing mice due to iron deficiency or hemolytic or myelopathic conditions. Treatment with EEIP at the dose of 100, 200 and 400mg/kg brought back the hemoglobin (Hb) content, RBC and WBC count more or less to normal levels significantly. This clearly indicates the protective action of EEIP at the dose of 100,200 and 400 mg/kg on the haemopoietic system.

The liver damage and loss of functional integrity of cell membrane was evidenced by elevated level of total cholesterol, TG, AST, ALT, ALP in serum of tumor inoculated animals. EEIP at the dose of 100,200 and 400 mg/kg significantly reversed these changes to normal. The biochemical examination of DLA inoculated animals showed marked changes indicating the toxic effect of the tumor. The normalization of these effects observed in the serum treated with EEIP at the dose of 100,200 and 400 mg/kg supported the potent antitumor effect of the EEIP.

Indian propolis extract has been standardized using a new simple, precise and reliable HPLC method in terms of Caffeic acid, Apigenin, Quercetin and CAPE. The polyphenols and flavonoids rich propolis extract exhibited better *in vitro* anticancer activity than pure CAPE, a

potent anticancer constituent of propolis. Anti tumor activity *in vivo* reveals that EEIP was effective on inhibiting the tumor progression, most likely because of synergistic activity of constituents present in the extract. However, the exact molecular mechanism by which EEIP mediates its antitumor activity is to be studied. From pesticidal analysis, hemolysis, plasma protein binding and acute toxicity studies it can be concluded that the EEIP is safe for internal use and can be considered for development of suitable formulation. Based on above promising results, further development of suitable formulation for CAPE and EEIP and its *in vivo* anti tumor study are in process.

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PART 2

Part 2

Indian propolis loaded Poly (ε-caprolactone) nanoparticles as a nanomedicine for improved anticancer efficacy

Introduction

Propolis, a natural honey bee product contains various polyphenols and flavonoids in which caffeic acid phenethyl ester is most active polyphenolic constituent responsible for anticancer activity [1]. Propolis of various regions is known to exhibit various activities including antifungal, antibacterial and anticancer activity [2, 3, 4]. Various *in vitro* and *in vivo* anticancer studies on propolis and its isolated constituents have been reported for anticancer activity [5, 6, 7]. Although propolis has beneficial anticancer activity on human cancer cell lines but it has some limitations such as it is high lipophilicity, poor solubility in water. The overall effect leads to low bioavailability and poor biological activity.

The solubility is an important factor on which drug dissolution, absorption and bioavailability are directly depending. The biological effects of drugs get affected because of its low solubility. Various approaches are reported in order to increase drug solubility which includes cyclodextrin complex formation solid dispersions or some chemical modifications. Various limitations associated with these approaches [8]. Polymeric nanoparticle system is one of the approaches established to overcome the limitations. The biodegradable polymeric nanoparticles have been investigated especially for achieving increased drug solubility, drug targeting to cancer cells due to their small particle size (10–1000 nm) by enhance permeability and retention effect, prolonging the biological activity by extending blood circulating time, and increasing drug concentration and residence time of drug at specific site of body [9].

Poly (ε-caprolactone) (PCL) is a biodegradable polymer most commonly used for pharmaceutical applications [10, 11, 12]. PCL has various advantages such as biocompatibility, biodegradation and applicability in formulation development [13]. PCL has been used extensively for developing nanoparticulate drug delivery systems. PCL has various advantageous properties such as high hydrophobicity and permeability and its biodegradation end product 6-hydroxycaproic acid is neutral, which does not disturb the pH balance [14]. It has been reported that the due to electrostatic interactions positively charged carriers non-specifically interact with a non target cells and negatively charged components of blood [15, 16]. For the tumour targeting, one of the approach is making the carrier particle surface negatively charge with hydrophilic poloxamers and poloxamines (Pluronic®) for reduction of such type of interactions [17]. Biodegradable and biocompatible materials including PCL and poly(dl-lactic-co-glycolic) have been investigated for encapsulation processes, but very few publications in the literature are related to encapsulation of propolis at the nanoparticle levels [18].

Quality by design (QbD) approach helps researchers to understand the effects of critical variables in formulation and process on the product quality. Design of Experiment (DoE) is a key factor involved in QbD. Nanoparticle formulations can be developed using DoE

approach to identify critical process parameters, reduce number of experiments, and study the interactions between formulation variables [8]

In addition, natural products are known for their activities through synergistic mechanisms so prompted by the above facts, the present study has been designed to develop standardized ethanolic extract of Indian propolis (EEIP) loaded PCL nanoparticles (denoted as EPL) using Design of Experiment approach to solve issue of poor water solubility, study synergized anticancer potency of EEIP, achieve controlled drug release and increased *in vitro* anticancer efficacy.

Materials and methods

Materials

Pluronic® F-68, PCL (poly-ε-caprolactone, mw 65,000 Da), and dialysis bag with a 12,000 molecular weight cut off, were purchased from Sigma-Aldrich Chemical Private Ltd (Bangalore, India). Acetone GR grade, sodium hydroxide and potassium dihydrogen phosphate were purchased from Merck India. All other chemical reagents used were of pharmaceutical grade.

Extraction and characterization of Indian bee propolis

10g of crude propolis was pre treated with hexane and ethyl acetate to remove wax and further extracted with ethanol by soxhlet method at 60^0C to obtain ethanolic extract of Indian propolis (EEIP), which was stored in 2-8^0 C and used for further evaluation. EEIP was standardized for CAPE using developed high performance liquid chromatography (HPLC) method.

Preparation of EEIP loaded polymeric nanoparticles

Briefly, EEIP was dissolved in 3 ml of acetone containing PCL polymer and Pluronic® F-68 (Table 1). Organic solution was injected into 10 ml distilled water at a rate of 10 ml/min under magnetic stirring at 2000 rpm for 1 h at 40°C. Resultant aqueous nanoparticle suspension was filtered through 0.45 μ filter to remove copolymer aggregates. Concentrations of PCL and Pluronic® F-68 (PF 68) were also optimized and selected to obtain stable suspension. Nanoparticle suspension prepared without Pluronic® was unstable because of aggregation of polymeric particles [9].

Design of experiments DoE approach

For determination of optimized nanoparticle formulation Design of experiment approach was used. PS and EE values were measured after collecting the nanoparticles. For the statistical design, Design Expert® Version 10.0 was used and PS and EE were selected as dependent variables while the amount of polymer (PCL) and amount of surfactant (PF 68) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of each system were performed.

Particle size analysis, Drug content and Encapsulation Efficiency (EE)

The PS of developed formulations was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The PS measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average PS was determined, expressed in terms of d (0.9) μm.

The concentration of active ingredients (DC and EE) in the polymeric nanoparticle formulation was measured by high performance liquid chromatography method (HPLC, Jasco UV 2057, Japan). A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 ml/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 μ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 μL, and the detection wavelength was set at 331 nm. Temperature was maintained at $25^0 \pm 2^0$ C. The nanoparticle solution was suitably diluted with alcohol prior to determination.

The percent DC was calculated according to the equation:
DC (%) = (Amount of EEIP in nanoparticle/ Amount of EEIP and polymer) × 100 (1)
EE was determined by separating non-encapsulated active ingredients from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4^0C. The sediment nanoparticles were disrupted with acetone to release the entrapped CAPE and EEIP; suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method, (Jasco UV 2057, Japan)
The percent EE was calculated using Equation (2).
EE (%) = Amount of EEIP entrapped in the vesicle/ Initial amount of the EEIP added × 100. (2)

Optimization by 3^2 factorial design

Preliminary experiments helped in understanding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PCL (X1) and PF 68 (X2) concentration were identified as crucial factors in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was adopted to optimize the nanoparticles composition while studying the effect of X1 and X2 (independent variables) on PS and EE (two dependent variables or responses) by obtaining a 3D response surface plot.

Zeta potential

The zeta potential study was carried out with the laser Doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments) at a temperature of 25°C.

Differential scanning calorimetry

The differential scanning calorimetry (DSC) thermograms of EEIP, PCL and EPL (vacuum-evaporated samples) were obtained using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples were (5 mg) heated in hermetically sealed aluminium pan with a heating rate of 10°C/min under a nitrogen atmosphere (flow rate 50 ml/min).

Fourier transform-infrared spectroscopy (FTIR)

FTIR spectra of EEIP, PCL and nanoparticle formulations were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan) upon mixing in dry KBr. Each sample was scanned in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of nanoparticle suspensions was studied using transmission electron microscopy (TEM). A drop of EPL was applied to a carbon film-covered copper grid. Most of the suspension was blotted from the grid with filter paper to form a thin film specimen. The unstained sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV and a magnification of 50000X.

***In vitro* drug release study**

Polymeric nanoparticle formulation (each 5 ml) was placed into a dialysis bag (cut off 12,000D).The bag was immersed into a beaker containing phosphate buffer (pH 7.4) at 37 ± 0.5 °C with continuous magnetic stirring at 100 rpm. At predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48 and 54 h) the sample (5 ml) was withdrawn maintaining sink condition. The samples were analyzed for EEIP content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of EEIP was plotted against time. Pure EEIP release was analyzed in a similar manner [19].

***In vitro* cytotoxicity studies**

Cytotoxic activity of EEIP and EPL was evaluated on human breast cancer MCF-7 and human colon cancer HT-9 cell line using sulforhodamine B (SRB) assay. The study performed as per the procedure described by Bothiraja et al, 2013 at Tata Memorial Center, Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Formulations were diluted in distilled water so as to obtain EEIP in the concentration ranges of 10, 20, 40 and 80μg/ml which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^3 cells per well in 96-well plates using in-situ fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37ºC with 100% relative humidity (RH), the growth medium was replaced with 100 μL of fresh medium containing various concentrations (10-80 μg/mL) of EEIP and EPL. The culture media without any drug formulation was used as a control. After 48 h incubation, assay was terminated by adding 50 μL of the cold TCA and incubated for 60 min at 4ºC. The media was removed and washed with sterile PBS and dried. 50 μL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation.

Cell growth (%) = (Average absorbance of the test well / Average absorbance of the control wells) ×100 [20].

Haemolysis study

The hemolytic effects of the EEIP and EPL were determined using the method described by love et al. briefly, fresh 10 ml blood collected from healthy donors and fibrinogen was removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. after removal of supernatant the precipitate diluted with aqueous NaCl solution (1:50 v/v) to get 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number represented a set of three tubes. To all of the tubes 2.5 mL of the red blood cell suspension was added. The tubes labeled 1 were diluted with 2.5 mL of distilled water as the hemolysis control (100% hemolysis) and the tubes labeled 2 were diluted with 2.5 mL of 0.9% isotonic NaCl solution as the non hemolysis control (0% hemolysis). The remaining tubes were diluted with EEIP and EPL samples corresponding to concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/mL of formulations and volume made up to 5 mL with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer [21]

Stability study

EPL was subjected to accelerated stability studies for three months as per ICH guidelines at a temperature of 25 ± 2^0 C and 60% RH. Optimized nanoparticles were analyzed for the change in PS and EE.

Results and discussion

In present study attempt has been made to develop EEIP encapsulated nanoparticulate formulations by considering its safety and anticancer efficacy on breast cancer and colon cancer cell lines. Nanotechnolgy based drug delivery systems has proven for improving the solubility, efficacy and safety of various drugs over the past few decades [22-24].

In this study, EEIP loaded polymeric nanoparticles have been developed and investigated as an intravenous nanocarriers in order to improve its solubility, to achieve control release and improve *in vitro* anticancer efficacy. The effect of the EEIP loaded polymeric nanoparticles composition on PS and EE was studied by design of experiments (DoE) approach.

Effect of polymer concentration on PS and EE

Initially effect of EEIP: PCL at different ratios (1:2.5/5/7.5/10) on particle size and encapsulation efficiency was studied. At 1:5 ratio minimum PS and maximum EE was observed. Further EEIP: PCL at ratios of (1:3.75/5/6.25) was studied. At all ratio results obtained was satisfactory to our requirement. Further detail studies considering surfactant concentrations, dependant and independent variables were done for final optimization of formulation batch.

PS, DC & EE

The mean EPL nanoparticles size was in the range of 190 ± 1 - 230 ± 2 nm and was strongly affected by the selected variables. The DC and EE were in the range of 92.36± 0.45 to 95.83± 0.45% and 62.66± 1.20 - 67.18± 1.80 % respectively for EPL (Table 1). A good fit (r^2 for PS = 0.9941 and EE = 0.8292 for ENP) was observed for the PS and EE the independent variables.

Drug delivery to specific sites of the body is influenced by size of the nanoparticles; smaller particles may tend to minimize the particle uptake by non targeted cells, including their premature clearance by the mononuclear phagocytic system [25].

Table1. The effect of various formulations (with different amounts of surfactant and polymer) on particle size and EE by 3^2 factorial design with coded levels and actual values of variables.

Batches EPL	Coded levels (X1, X_2)	Amt. of PCL (mg; X_1)	Amt of PF 68 (mg; X_2)	Drug Content (%)	Nanoparticle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)
F1	+1, +1	62.50	100.00	94.37± 0.90	230± 2	65.71± 1.20
F2	+1, 0	62.50	75.00	94.73± 0.45	226± 2	64.33± 1.20
F3	+1,-1	62.50	50.00	95.83± 0.45	220± 2	64.23± 1.80
F4	0, +1	50.00	100.00	93.42± 0.90	203± 1	65.42± 1.20
F5	0, 0	50.00	75.00	93.61± 0.75	200± 2	66.61± 1.80
F6	0, -1	50.00	50.00	93.80± 0.75	199± 2	67.18± 1.80
F7	-1, +1	37.50	100.00	92.62± 0.45	198± 3	63.62± 1.80
F8	-1, 0	37.50	75.00	92.62± 0.90	195± 2	63.20± 1.45
F9	-1, -1	37.50	50.00	92.36± 0.45	190± 1	62.66± 1.20

Values were presented as mean ± SD (n = 3).

Optimization of EPL nanoparticles by 3^2 factorial design

PS and EE are the major important factors considered in formulation development of nanoparticles. During the preliminary study, assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of polymer and surfactant (Table 1). EEIP content was kept constant. As per 3^2 factorial design, nine different batches were prepared for each system. The responses of these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using Design Expert® Version 10.0. The results of multiple regression analysis of the obtained data are summarized in Table 2.

From the factorial design study of EPL (Table 2), we observed that positive coefficients of the main terms X1 and X2 indicated a favorable effect on the mean particle size and EE with the PCL and PF68. The PCL had a linear effect on the PS and curvilinear effect on EE while PF 68 had linear effects on both PS and EE as seen in surface plot (Figure 1).

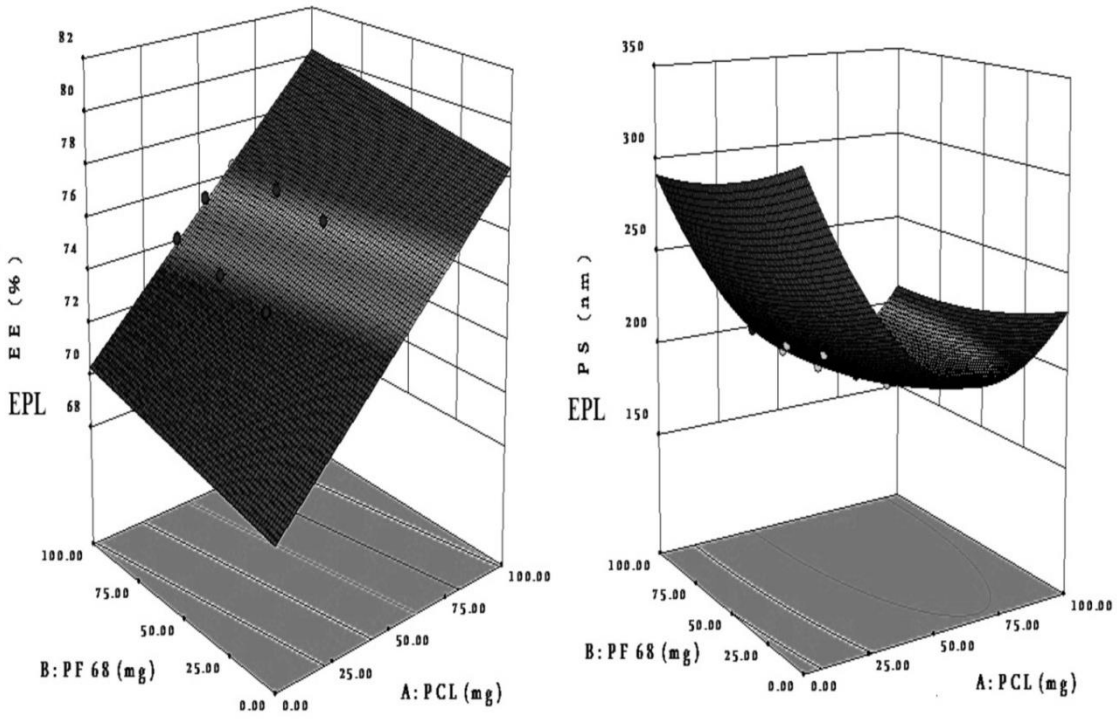
Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.700 was obtained with F3 for EPL so F3 for EPL was selected as an optimized formulation and further evaluated for various parameters.

Table 2. The results of multiple regression analysis of the obtained data from Design Expert® Version 10

Sr. No	Final Equation in Terms of Coded Factors		
	EE	**PS**	
1	+63.21	+191.06	
2	+22.23	+13.33	* X1
3	+0.39	+7.67	* X2
4	+1.04	+4.00	* X1X2
5	-39.12	+146.67	* $X1^2$
6	+0.36	-1.33	* $X2^2$

Number	PLGA	PVA	EE	PS	Desirability
EPL	65.564	50.000	66.337	201.116	0.700

Figure 1. Response surface plot showing effects of amount of PCL and PF 68 (independent variables) on dependent variables PS and EE for EPL



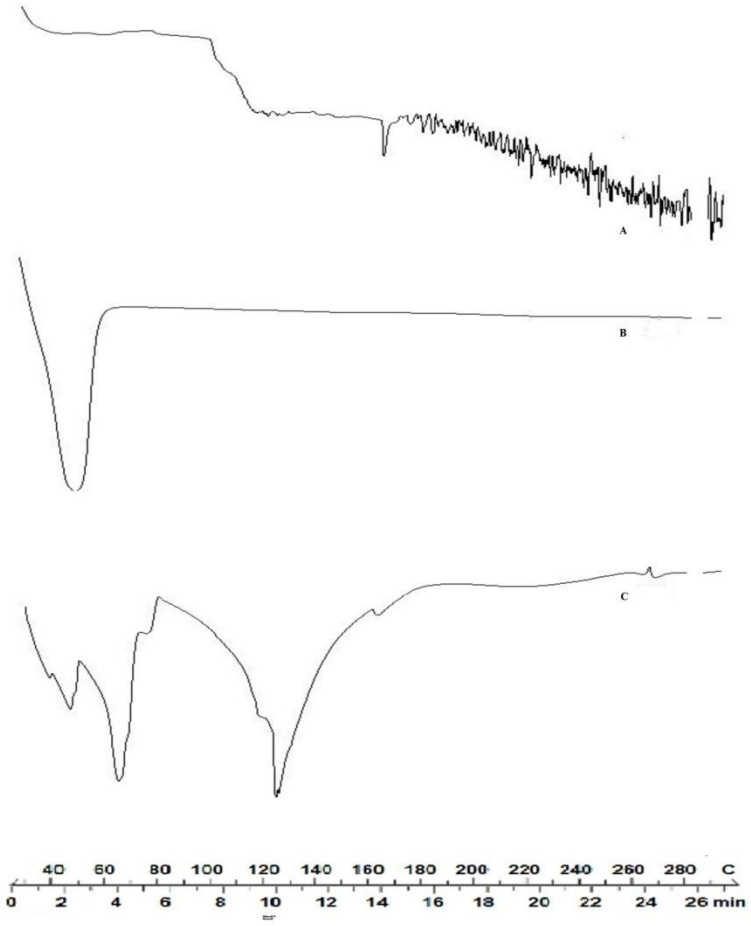
Zeta potential

Zeta potential is essential parameter gives information about surface charges surface charges which has direct influence on colloidal stability and interaction with physiological body cells. The EPL showed negative zeta potential (–26.44 ± 0.10 mV) which may be due to presence of PF 68 on surface. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability and blood clearance.

Differential scanning calorimetry

Study was performed for the free EEIP, PCL and EPL (Figure 2) in order to determine the molecular state of the EEIP. A sharp melting transition of free EEIP was observed at 127.48 °C with \H 38.01 J/g shows transit crystallinity. In EPL thermogram, the free EEIP peaks were disappeared indicating molecular dispersion of EEIP inside polymeric nanoparticles.

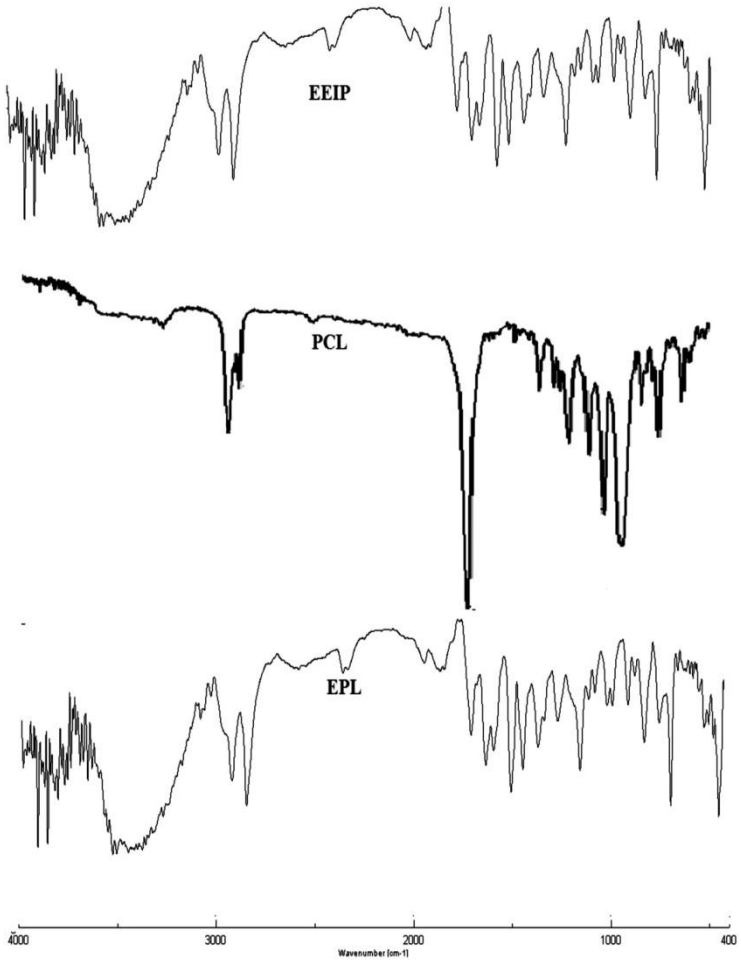
Figure 2. Comparative DSC thermo grams of EEIP (A), PCL (B), EPL (C)



Fourier transform-infrared spectroscopy (FTIR*)*

Figure 3 shows FTIR spectra of pure EEIP (A), PCL (B) and EPL (C). It can be seen from the FT-IR spectrum of free EEIP showed various peaks at 3571.52 cm−1, 3541.63 cm−1, 3485.7 cm−1 and 3333 cm−1 that were assigned to –OH stretching. Band obtained at 3083.62 cm -1 was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm−1, 1594.8 cm−1, and 1164.7 cm−1 were attributed to C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PCL the bands at 2940 cm^{-1} and 2860 cm^{-1} were C–H hydroxyl groups asymmetric stretching and C-H hydroxyl groups symmetric stretching respectively. 1722 cm^{-1} is assigned to C=O stretching vibrations of the ester carbonyl group. The absorption at 1238 cm^{-1} is assigned to C-O-C asymmetric stretching and 1160 cm^{-1} is C-O-C symmetric stretching. However, in the FTIR spectra of EPL the major peaks at 3571.52 cm−1, 3541.63 cm−1 were assigned to –OH stretching. Band obtained at 3083.62 cm -1 was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm−1, 1594.8 cm−1, and 1164.7 cm−1 were significantly decreased by its intensity and the presence of these characteristic peaks is a confirmation of EEIP encapsulation on PCL nanoparticles successfully.

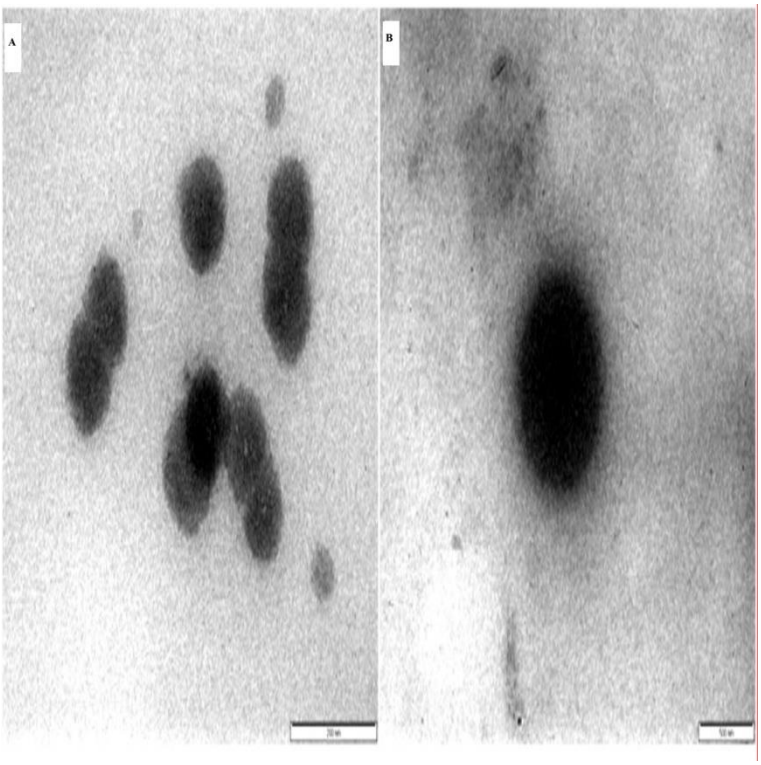
Figure 3. Comparative FTIR spectra of pure EEIP, PCL, and EPL



Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of the EPL was assessed using transmission electron microscope (TEM) from which it can be seen that the nanoparticles were freely dispersed and spherical in shape (Figure 4). Little bigger size nanoparticles were observed in TEM than that tested by laser diffraction technique. Because of low melting points of PF 68 (~55 °C) and PCL (~60 °C), nanoparticles may undergoes the melting due to high energy electron beam in TEM. Melting might cause expansion to certain level and nanoparticles seem bigger in TEM.

Figure 4. Transmission electron microscopy images of EPL (A&B)

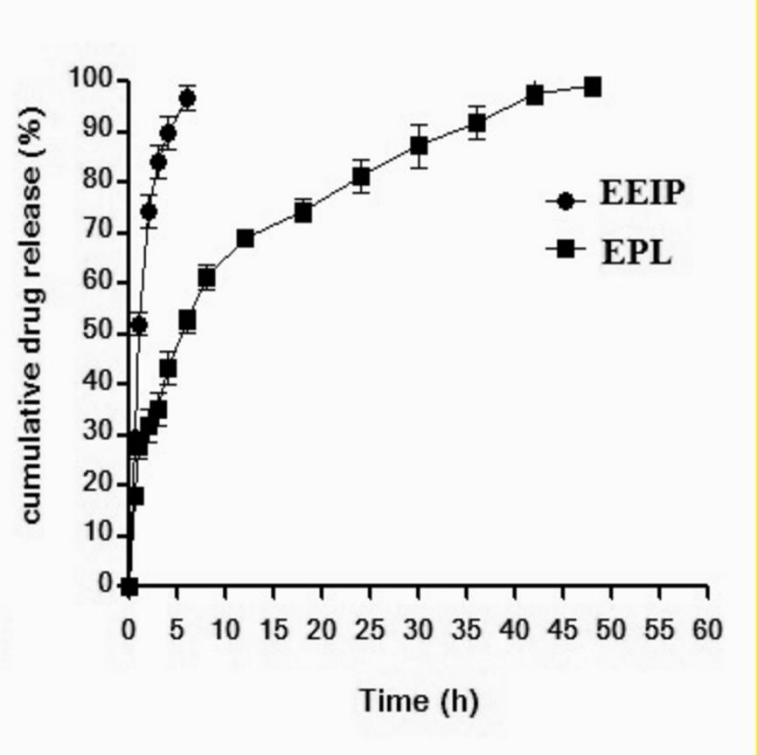


***In vitro* release study**

In vitro release of the EEIP from solution of EPL was investigated by diffusion bag technique. As EPL was developed aiming at its intravenous administration, the release studies were conducted in phosphate buffer saline (pH 7.4) at 37°C. Figure 5 revealed that EEIP could freely diffuse in its solution form causing 96.79% drug release within 6 h respectively. However, EEIP release from polymeric nanoparticles showed a biphasic pattern with initial burst release (18.00%) within the first 1 h followed by sustained release up to 48 h for EPL.

Erosion, diffusion and degradation are three basic mechanisms contributed for the release of a loaded drug from polymeric nanoparticles. For developed system any or all three mechanisms may be involved in drug release. During the *in vitro* release study, the initial burst release may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a controlled release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Figure 5 the release profile of EEIP from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by a initial rapid release followed by a slower continuous release phase over 48 h.

Figure 5. *in vitro* release of pure EEIP and EEIP from EPL



***In vitro* anticancer activity**

The *in vitro* anticancer activity of EPL was investigated and compared with EEIP against human breast cancer MCF-7 cells and human colon cancer cells HT-29 using *in vitro* SRB assay.

The results illustrated in Table 3, Figure 6 indicated that EPL displayed superior anticancer activity than EEIP on breast cancer MCF-7 cells. The total growth inhibition concentration of EPL was observed 20.5 µg/ml while for EEIP it was 30.1 µg/ml.

In case of colon cancer HT-29 cells lines Table 3, Figure 7 indicated that EPL displayed superior anticancer activity than EEIP. The total growth inhibition concentration of EPL was observed 31.00 µg/ml and for EEIP 39.9 % µg/ml.

The enhanced anticancer efficacy may be attributed to greater cellular uptake of EPL via phagocytosis or the fusion process which resulted in the enhanced permeability of the cell membrane to EEIP that allows sufficient drug concentration inside the cells. Therefore, EPL might serve as a potential nanocarrier to improve *in vitro* anticancer activity of EEIP. The lower anticancer activity of free EEIP in solution may be due to its efflux by P-glycoprotein pumps. The GI50 values for EEIP and EPL were observed less than 10µg/ml indicates potent anticancer nature on both cell lines.

Table 3. TGI and GI_{50} values of EEIP, EPL and ADR on MCF-7 breast cancer and HT-29 colon cancer cell line.

Samples	MCF-7 cell line TGI (µg/mL)	MCF-7 cell line GI_{50} (µg/mL)	HT-29 cell line TGI (µg/mL)	HT- 29 cell line GI_{50} (µg/mL)
EEIP	30.10	< 10	39.90	16.50
EPL	20.50	< 10	31.00	< 10
ADR	< 10	< 10	< 10	< 10

Values were presented as mean ± SD (n = 3).

TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produces 50% inhibition of cells, MCF 7 and HT-29 cell line.

Figure 6. *In vitro* cytotoxicity study on MCF-7 Cell lines (A) MCF-7 control (B) Positive control ADR (C) EEIP (D) EPL Treated

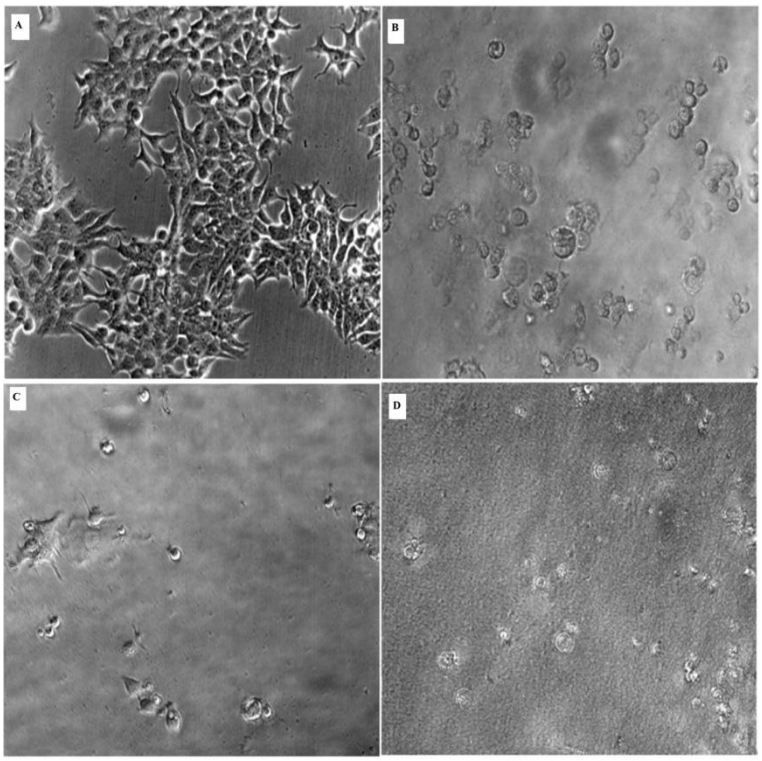
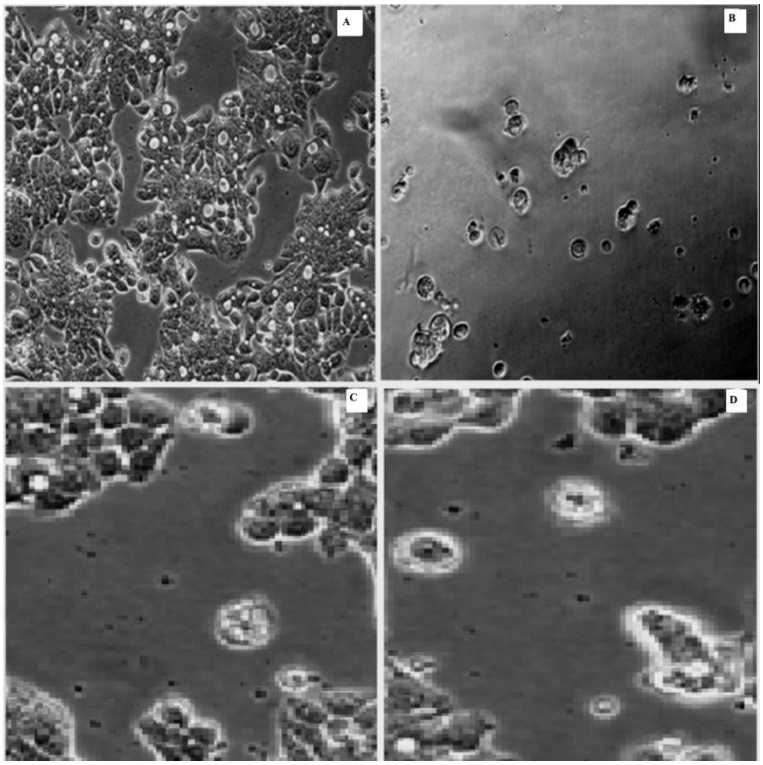


Figure 7. *In vitro* cytotoxicity study on HT-29 Cell lines (A) MCF-7 control (B) Positive control ADR (C) EEIP (D) EPL Treated



Hemolysis study

For *in vivo* application it is necessary to evaluate the biosafety by hemolysis study. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate for up to 1.5 mg/mL concentration of EEIP and EPL were 2.8 and 2.7, % respectively. The acceptable hemolysis rate (less than 3%) [21] Shown denotes formulations having non hemolytic property up to 1.5 mg/mL and may be considered as biosafe for internal use.

Stability studies

EPL was subjected to stability study at 40 ± 2°C/75 ± 5% RH. No significant difference in EE and PS were observed over the period of three months as compared to initial values of freshly prepared nanoparticle formulations. ($p > 0.05$).

Table 4. Accelerated stability study of EPL for 3 months

Time (Months)	PS (nm)	Percentage of EE
	EPL	EPL
Initial	201±3	64.23±1.96
1	202±3	64.00±0.88
2	202±3	63.60±1.54
3	203±3	63.41±1.28

Values were presented as mean ± SD (n = 3). Data was analyzed by one way ANOVA followed by Dunnett's multiple comparison test with $p < 0.05$.

Conclusion

We have investigated nanoparticulate formulation composed of PCL and PF-68 as an injectable nano carrier for the delivery of an anticancer bioactive, EEIP. Developed formulation displayed smaller particle size, excellent encapsulation efficiency and control release with better stability. EPL demonstrated increase in anticancer efficacy as compared to EEIP. EPL demonstrated higher *in vitro* anticancer activity in human breast cancer MCF-7 cells and human colon cancer HT-29 than its free form, which may leads to reduction in dose as well as cost. Developed formulation can be considered as an alternative dosage form for EEIP to improve the biopharmaceutical properties and to enhance therapeutic efficacy in cancer chemotherapy.

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PART 3

Part 3

Indian propolis loaded Folic acid conjugated PLGA nanoparticles: Formulation development, characterization, *in-vitro* and *in-vivo* anticancer study

Introduction

Propolis, a resinous material collected by honey bees specifically *Apis mellifera* from the various plant parts, exudates and buds is known for its nutritional and medicinal properties obtained from apiculture.[1] Apart from use of propolis as a nutritional and healthy food constituent, its use in human health in folk medicine [2] has been reported from ancient time. Propolis of various regions is known to exhibit various activities including antifungal, antibacterial and anticancer activity.[3, 4, 5] Various *in vitro* and *in vivo* anticancer studies on propolis and its isolated constituents have been reported for anticancer activity.[6,7,8] Caffeic acid phenethyl ester (CAPE), the most active chemical constituent of honeybee propolis is chemically poly phenolic compound.[9,10] Although propolis has beneficial anticancer activity on human cancer cell lines but it has some limitations such as it is highly lipophilicity of various polyphenols and flavonoids which are responsible for anticancer activity , poor solubility in water. The overall effect leads to low bioavailability and poor biological activity. Drug dissolution, absorption and bioavailability are directly depending upon solubility factor. The potential effects of drugs get reduced due to its low solubility. Various approaches are reported in order to increase drug solubility which includes solid dispersions, cyclodextrin complex formation or some chemical modifications. There are various limitations associated with these approaches.[11] Polymeric nanoparticle system is one of the approaches established to overcome these limitations. The biodegradable polymeric nanoparticles have been investigated especially for achieving increased drug solubility, drug targeting to cancer cells due to their small particle size (10–1000 nm) by enhanced permeability and retention effect (EPR), prolonging the biological activity by extending blood circulating time, and increasing drug concentration and residence time of drug at specific site of body.[12]

Although a number of different biodegradable polymers have been investigated for formulating nanoparticles, Poly (D, L-lactide-co-glycolide) (PLGA) have been widely used for controlled drug delivery systems as a carrier for anticancer agents.[13] Further, Folic acid, a water soluble vitamin which is oxidized form of folate is used as a ligand due to its efficient internalization via folate receptor mediated endocytosis for tumor targeting. Folic acid is abundantly expressed in various types of human tumors such as breast, ovarian and prostate cancer. But in case of normal tissues it is minimally distributed. Therefore, Folic acid can serve as potential tumor marker and a functional tumor-specific receptor.[14, 15, 16]

Limited studies have been reported on Indian propolis to improve its solubility and efficacy through nano technology based drug delivery systems, however these studies have failed to study the detailed characterization of formulations. There are only few reports for CAPE or Indian propolis formulations targeting anti cancer activity.[17, 18] In addition, natural products are known for their activities through synergistic mechanisms so prompted by the above facts, the present study has been designed to develop standardized ethanolic extract of

Indian propolis (EEIP) loaded PLGA– folic acid conjugated polymeric nanoparticles (denoted as EPFA) using Design of Experiment approach to solve issue of poor water solubility, study synergized anticancer potency of EEIP, achieve controlled drug release and increased *in vitro* and *in vivo* anticancer efficacy.

Materials and methods

Materials

PLGA with a copolymer ratio of d-l-lactide to glycolide of 50:50 (Mw 40,000–100,000 g/mol), poly(vinyl alcohol) PVA (87–89% hydrolysis degree and molecular mass 12,000–13,000 g/mol, dialysis bag with a 12,000 Da molecular weight cut off, 1,3,Dicyclo-hexyl carbodiimide (DCC), N-hydroxysulfosuccinamide (Sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Bangalore, India. Acetone GR grade, sodium hydroxide, potassium dihydrogen phosphate, dichoromethane (DCM) and ethanol were purchased from Merck India. All other chemical reagents used were of analytical grade.

Extraction and characterization of Indian stingless bee propolis

10g of crude propolis was pre treated with hexane and ethyl acetate to remove wax and further extracted with ethanol by soxhlet method at 60^0C to obtain ethanolic extract of Indian propolis (EEIP), which was stored in 2-8^0 C and used for further evaluation. EEIP was standardized for CAPE using developed reverse phased high performance liquid chromatography (RP-HPLC) method.

Preparation of EEIP loaded polymeric nanoparticles

EEIP loaded PLGA nanoparticles (denoted as ENP) were prepared by oil in water (o/w) single emulsion solvent evaporation method described by Song et al., 2008 with minor modifications. Formulation batches were prepared as per composition shown in Table 1. The amount of EEIP (10mg) kept constant. The organic phases consisted of PLGA dissolved in dichloromethane (DCM), EEIP were dissolved in ethanol. This organic solution was emulsified with the 5 mL aqueous solution of PVA as per different proportions shown in Table 1 by sonication (output power 70W, power of 80%, and 2 minutes) using a probe sonicator (U 200 S Control, Ika labrortech staufen, Germany) over an ice bath. The o/w single emulsions were stirred overnight on a magnetic stirrer at 1500 rpm at room temperature for evaporation of organic phase. The resulting particles were collected by centrifugation at 9000x rpm for 40 min (eppendorf Centrifuge 5424 R), washed three times with ultra-pure water to remove excess PVA. Polymeric nanoparticles were separated from the free active ingredients or polymer agglomerate by filtration through a 0.45 μm membrane filter and then lyophilized. Lyophilized nanoparticles were stored at -80^0 C until used. [19]

Effect of polymer concentration particle size (PS) and encapsulation efficiency (EE)

The effect of polymer concentration was studied by studying effects of different EEIP: PLGA ratio upon PS and EE.

Design of experiments DoE approach

A DoE approach was used to determine the optimized nanoparticle formulation. After collecting the nanoparticles, particle size (PS) and encapsulation efficiency (EE) values were measured. For the statistical design, Design Expert® Version 10.0 was used and PS and EE were selected as dependent variables while the amount of polymer (PLGA) and amount of surfactant (PVA) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of each system were performed.

DC, PS and EE analysis

The PS of developed formulations was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The PS measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average PS was determined, expressed in terms of d (0.9) μm.

The concentration of EEIP (DC and EE) in the polymeric nanoparticle formulations was measured by HPLC method. A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 mL/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 μ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 μL, and the detection wavelength was set at 331 nm. Temperature was maintained at $25^0 \pm 2^0$ C. The nanoparticle solution was suitably diluted with alcohol prior to determination.

The percent DC was calculated according to the equation:
DC (%) = (Amount of EEIP in nanoparticle/ Amount of EEIP and polymer) × 100 (1)
EE was determined by separating non-encapsulated active ingredients from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4^0C. The sediment nanoparticles were disrupted with acetone to release the entrapped EEIP; suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method, (Jasco UV 2057, Japan)
The percent EE was calculated using Equation (2).
EE (%) = Amount of EEIP entrapped in the vesicle/ Initial amount of the EEIP added × 100. (2)

Optimization by 3^2 factorial design

Preliminary experiments helped in understanding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PLGA (X1) and PVA (X2) concentration were identified as factors crucial in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was adopted to optimize the nanoparticles composition while studying the effect of X1 and X2 (independent variables) on PS and EE (two dependent variables or responses) by obtaining a 3D response surface plot.

Surface conjugation with folic acid

The surface modification was carried out by previously published method described by Das and Sahoo et al., 2012 with minor modification. Folic acid was reacted at stoichiometric molar ratio of Folic acid: DCC: NHS = 1:1.2:2 in dimethyl sulphoxide (DMSO) for 6 hrs at 50^0C. N, N- dicyclohexylurea (DCU) formed in reaction was removed by filtration. Formed product was allowed to react over night with ethylene diamine in presence of pyridine which acts as catalyst. The aminated folate formed was precipitated out by adding excess cold acetonitrile, separated by vacuum filtration. For conjugation of aminated folate onto the surface of optimized ENP, 20 mg of ENP was dispersed in 10 mL of phosphate buffer solution (PBS) (0.02 M, pH 7.4). 250 µl of EDC (2 mg/mL) and NHS (2 mg/mL) each were added to the above suspension followed by agitating for 2 hrs at room temperature using a magnetic stirrer. By ultracentrifugation at 20,000 rpm for 20 min at 4^0C (eppendorf Centrifuge 5424 R), excess of unreacted EDC and NHS were removed and activated nanoparticles were recovered. For final conjugation, activated nanoparticles were dispersed in 4 mL of PBS (0.02 M, pH 7.4) followed by addition of 200 µl of aminated folate solution (1mg/mL in PBS). The solution was again agitated for 2 hrs at room temperature and excess of unconjugated folate was removed by ultracentrifugation and lyophilized for further use. Conjugation was confirmed by Fourier transform-infrared spectroscopy (FTIR) analysis.[20]

Zeta potential

The zeta potential was measured with the laser Doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments, UK) at a temperature of 25^0C.

Differential scanning calorimetry (DSC)

DSC thermograms of EEIP and EPFA (vacuum-evaporated samples) were obtained using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples were (5 mg) heated in hermetically sealed aluminium pan with a heating rate of 10^0C/min under a nitrogen atmosphere (flow rate 50 mL/min).

Fourier transform-infrared spectroscopy (FTIR)

FTIR spectra of EEIP, PLGA, Folic acid and nanoparticle formulations were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan) upon mixing in dry KBr. Each sample was scanned in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of nanoparticle suspensions was studied using transmission electron microscopy (TEM). A drop of ENP and EPFA suspension was applied to a carbon film-covered copper grid. Most of the suspension was blotted from the grid with filter paper to form a thin film specimen. The unstained sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV and a magnification of 50000X.

***In vitro* drug release study**

The *in vitro* release of EEIP and EPFA from the nanoparticle formulations were carried out in phosphate-buffer saline (pH 7.4) using dialysis bag diffusion technique. Polymeric nanoparticle formulations equivalent to 2 mg of EEIP and 2 mg EEIP solution (2 mg/mL in 50% w/w mixture of PEG 400 and water) as control was were placed into a dialysis bag (cut off 12,000D).The bag was immersed into a beaker containing in 50 mL of release medium with continuous magnetic stirring at 100 rpm at 37 $\pm$ 0.5 ^{0}C. At predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48, 54 and 60 h) the sample (5 mL) was withdrawn maintaining sink condition. The samples were analyzed in triplicate for EEIP content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of EEIP was plotted against time. Pure EEIP release was analyzed in a similar manner. [21]

***In vitro* cytotoxicity studies**

Cytotoxic activity of EEIP and EPFA were evaluated on human breast cancer cell line MCF-7 using sulforhodamine B (SRB) assay at Tata Memorial Center, Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Formulations were diluted in distilled water so as to obtain EEIP in the concentration ranges of 10, 20, 40 and 80μg/mL which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^{3} cells per well in 96- well plates using in-situ fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37ºC with 100% relative humidity (RH), the growth medium was replaced with 100 μL of fresh medium containing various concentrations (10-80 μg/mL) of EEIP and EPFA. The culture media without any drug formulation was used as a control. After 48 h incubation, assay was terminated by adding 50 μL of the cold TCA and incubated for 60 min at 4ºC. The media was removed and washed with sterile PBS and dried. 50 μL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation.

Cell growth (%) = (Average absorbance of the test well / Average absorbance of the control wells) ×100. [22]

Haemolysis study

The hemolytic effects of the EEIP and EPFA were determined using the method described by love et al. briefly, fresh 10 ml blood collected from healthy donors and fibrinogen was removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. after removal of supernatant the precipitate diluted with aqueous NaCl solution (1:50 v/v) to get 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number represented a set of three tubes. To all of the tubes 2.5 mL

of the red blood cell suspension was added. The tubes labeled 1 were diluted with 2.5 mL of distilled water as the hemolysis control (100% hemolysis) and the tubes labeled 2 were diluted with 2.5 mL of 0.9% isotonic NaCl solution as the non hemolysis control (0% hemolysis). The remaining tubes were diluted with EEIP and EPFA samples corresponding to concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/mL of formulations and volume made up to 5 mL with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer.[23]

In vivo anticancer study

Selection grouping and acclimatization of laboratory animals and Induction of cancer using DLA cells

The study protocol was approved by the Institutional Animal Ethics Committee (protocol number: CPCSEA/QA/06/2016). Male Swiss albino mice (20-25 gm) were used for the study. They were acclimatized to laboratory environment at (temperature 25 ± 2°C) and 12 h dark /light cycle with diet and water. Dalton's Lymphoma ascites (DLA) cells were supplied by Amla cancer research center, Trissur, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by intraperitoneal (i.p) transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilutions were made so that total cell count should be $1x10^6$ .Tumor growth in the mice was allowed for minimum seven days before starting treatments.

Treatment Protocol

Animals were divided in to fifteen groups of six each. G2- G15 was injected with DLA cells (1×10^6 cells per mouse) intraperitonially (i.p.).

Group 1(G1) served as the normal control, Group 2 (G2) served as the tumor control. Group 1 and 2 receives normal diet and Water.

Group 3 (G3) served as the positive control and was treated with injection 5-fluorouracil at 20 mg/kg body weight i.p.

Group 4 (G4), Group 5 (G5) and Group 6 (G6) were administered EEIP at a dose of 100 mg/kg , 200 mg/kg and 400 mg/kg body weight i.p. respectively.

Group 7 (G7), Group 8 (G8) and Group 9 (G9) were administered EPFA at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight i.p. respectively.

Evaluation of anticancer activity

Treatment was given after 24 h of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital plexus bleeding and the following parameters were evaluated. Hematological parameters like WBC, RBC count , Hb content, platelet count and packed cell volume; serum enzyme and lipid profile which include total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP); and derived parameter like body weight, life span (%) and

cancer cell count were evaluated. The results are expressed as mean ± S.E.M. The evaluation of the data was done using one way ANOVA followed by Newman-Keul's multiple comparison test; $p< 0.01$ implied significance. (Unnikrishnan and Kuttan, 1990; Sathiyanarayanan et al., 2006; Ronald, 1995; Prasad and Giri, 1994)

Stability study

EPFA were subjected to accelerated stability studies for three months as per ICH guidelines at a temperature of 25 ± 2^0 C and 60% RH. Optimized nanoparticles were analyzed for the change in PS and EE (Table 8).

Results and discussion

Propolis is a natural bee hive product which majorly contains various polyphenols and flavonoids and reported to possess potential antitumor effect on breast cancer cells. CAPE is one of the most potential constituent of propolis, however the isolation and purification process to obtain pure CAPE and some other chemical constituents is tedious, time consuming and costly. Moreover the synergistic effects of diverse chemical constituents of alternative medicines are well known compared to pure isolated compounds. So in present study attempt has been made to develop and compare nanoparticulate formulations of EEIP by considering its safety and anticancer efficacy on breast cancer.

In this study, EEIP loaded polymeric nanoparticles have been developed and investigated as injectable nano carriers in order to improve solubility, *in vitro* and *in vivo* anticancer efficacy. The effect of the EEIP loaded polymeric nanoparticles composition on PS and EE was studied by design of experiments (DoE) approach.

Effect of polymer concentration PS and EE

Initially effect of EEIP: PLGA at different ratios (1:2.5/5/7.5/10) on particle size and encapsulation efficiency was studied. At 1:5 ratio minimum PS and maximum EE was observed. Further EEIP: PLGA at ratios of (1:3.75/5/6.25) was studied. Results obtained were satisfactory to our requirement. Further detail studies considering surfactant concentrations, dependant and independent variables were done for final optimization of formulation batch.

DC, PS & EE

Mean ENP nanoparticles size was in the range of 178 $\pm$ 5 to 205 $\pm$ 5 nm and was strongly affected by the selected variables. The DC and EE were in the range of 90.76–94.99 % and 73.16–76.37 % respectively (Table 1). A good fit (r^2 for PS = 0.9971 and EE = 0.9809) was observed for the PS and EE the independent variables.

Table 1. The effect of various formulations (with different amounts of surfactant and polymer) on PS and EE by 3^2 factorial design with coded levels and actual amount values of variables.

Batches ENP	Coded levels ($X1$, X_2)	Amt. of PLGA (mg; X_1)	Amt of PVA (mg; X_2)	Drug Content (%)	Nanoparticle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)

F1	+1, +1	62.50	100.00	93.66± 0.77	178± 5	76.37± 1.89
F2	+1, 0	62.50	75.00	94.99± 1.55	180± 4	76.20± 1.96
F3	+1,-1	62.50	50.00	93.13± 1.25	183± 4	75.83± 1.44
F4	0, +1	50.00	100.00	93.12± 1.40	184± 4	75.42± 1.44
F5	0, 0	50.00	75.00	92.11± 1.00	185± 5	74.61± 1.58
F6	0, -1	50.00	50.00	91.84± 1.25	191± 4	74.18± 1.44
F7	-1, +1	37.50	100.00	91.42± 1.25	199± 4	74.16± 1.96
F8	-1, 0	37.50	75.00	90.82± 0.98	200± 5	73.62± 1.89
F9	-1, -1	37.50	50.00	90.76± 0.98	205± 5	73.16± 1.89

Values were presented as mean ± SD (n = 3).

Optimization of ENP nanoparticles by 3^2 factorial design

PS and EE are the major important factors considered in formulation development of nanoparticles. During the preliminary study, assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of polymer and surfactant (Table 1). EEIP content was kept constant. As per 3^2 factorial design, nine different batches were prepared for each system. The responses of these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using Design Expert® Version 10.0. The results of multiple regression analysis of the obtained data are summarized in Table 2.

From the factorial design study of ENP (Table 2), it was observed that positive coefficients of the main terms X1 and X2 for EE and negative coefficients of the main terms X1 and X2 for PS indicated a favorable effect on the mean particle size and EE with the PLGA and PVA. The PLGA and PVA had a linear effect on EE and curvilinear effect on PS as seen in response surface plot (Figure 1). Smaller particles were obtained at low PLGA content, probably due to high distribution efficiency of internal phase into external phase. Increase in the viscosity of internal phase with increased amount of PLGA also provides resistance for mass transfer during diffusion of internal phase into the external phase leading to particle enlargement.

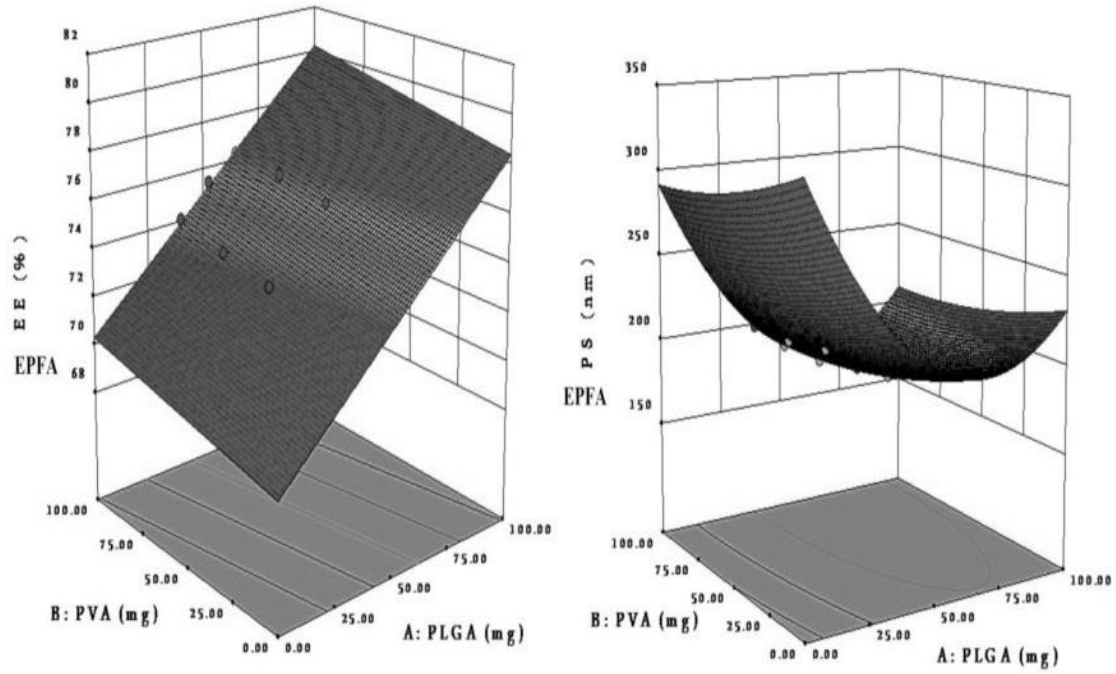
Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.941 was obtained with F1 for ENP having acceptable PS and EE, so F1 for ENP was selected as an optimized formulation and further evaluated for various parameters.

Table 2. The results of multiple regression analysis of the obtained data

Sr. no	Final Equation in Terms of Coded Factors		
	EE	**PS**	
1	+74.38	+190.22	
2	+4.97	-43.00	* X1
3	+0.93	-12.67	* X2
4	-	+2.00	* X1X2
5	-	+66.67	* $X1^2$
6	-	+6.67	* $X2^2$

Number	PLGA	PVA	EE	PS	Desirability
ENP	62.500	100.000	76.546	178.139	0.941

Figure 1. Response surface plot showing effects of amount of PLGA and PVA (independent variables) on dependent variables PS and EE for EPFA



Zeta potential

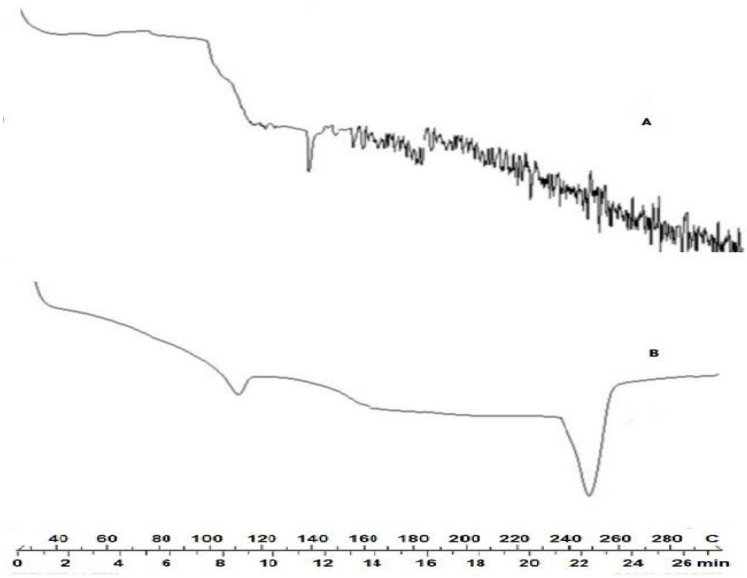
Zeta potential is essential parameter that gives information about surface charges which has direct influence on colloidal stability and interaction with physiological body cells. ENP showed negative zeta potential –17.71 ± 0.51 mV which may be due to presence of polyvinyl alcohol on surface. EPFA showed slight shifting of zeta potential towards positive

(–13.11 ± 0.88 mV) which may be due to surface modification and amide bond of folic acid. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability and blood clearance.

Differential scanning calorimetry

DSC study was performed for the free EEIP and EPFA (Figure 2) in order to determine the molecular state of the EEIP. In case of EEIP due to various polyphenols and flavonoids various melting transitions were observed. A sharp melting transition of was also observed at 127.02 °C with \H 17.59 J/g. In EPFA thermogram, the free EEIP peaks were disappeared indicating molecular dispersion of EEIP inside polymeric nanoparticles.

Figure 2. Comparative DSC thermo grams of EEIP (A) and EPFA (B)



Fourier transform-infrared spectroscopy (FTIR)

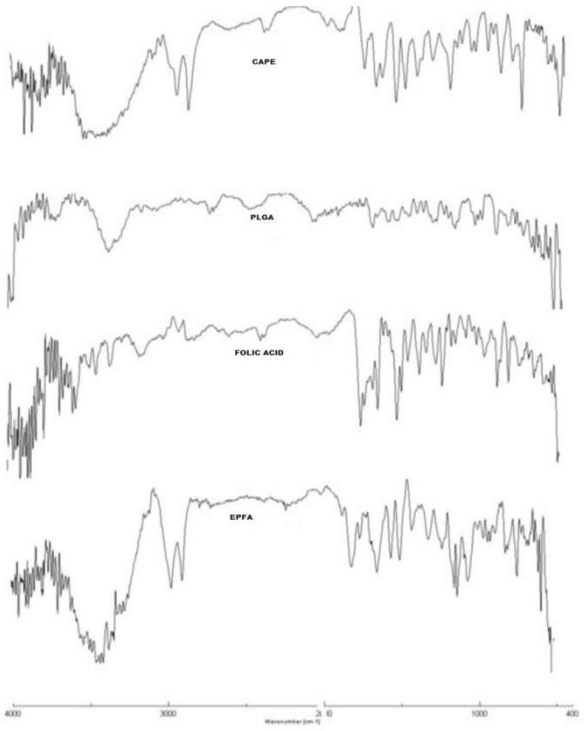
Figure 3 shows FTIR spectra of pure EEIP (A), PLGA (B), Folic acid (C) and EPFA (D). It can be seen from the FT-IR spectrum of free EEIP showed various peaks at 3571.52 cm^{-1}, 3541.63 cm^{-1}, 3485.7 cm^{-1} and 3333 cm^{-1} that were assigned to –OH stretching. Band obtained at 3083.62 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm^{-1}, 1594.8 cm^{-1}, and 1164.7 cm^{-1} were attributed to C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PLGA and nanoparticles the bands at 2995 cm^{-1} and 2949 cm^{-1} were C–H stretch of CH_2 and C–H stretch of –C–H–, respectively. A band at 1772 cm^{-1} and 1751 cm^{-1} was assigned to the stretching vibration of C=O of ester bond (strong and narrow) and 1186– 1087 cm^{-1} was attributed to C–O stretching, which belongs to the characteristic peaks of PLGA molecule.

However, in the FTIR spectra of EPFA, the major peaks at 3571.52 cm^{-1}, 3541.63 cm^{-1} were assigned to –OH stretching. Band obtained at 3083.62 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm^{-1}, 1594.8 cm^{-1}, and 1164.7 cm^{-1} were significantly

decreased by its intensity and the presence of these characteristic peaks is a confirmation of EEIP encapsulation on PLGA nanoparticles successfully.

Conjugation of folic acid to PLGA nanoparticles was confirmed by the amide bond peak at 1634.38 cm^{-1} in EPFA which clearly indicates the formation of amide bond following conjugation

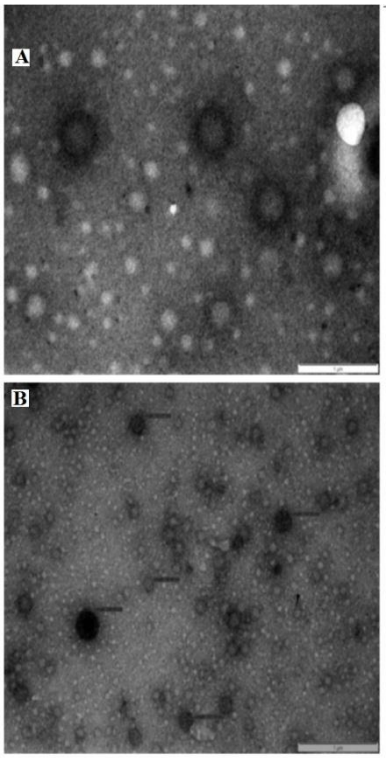
Figure 3. Comparative FTIR spectra pure EEIP (A), PLGA (B), Folic acid (C) and EPFA (D)



Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of the ENP and EPFA was assessed using transmission electron microscope (TEM) from which it can be seen that the nanoparticles were freely dispersed and spherical in shape (Figure 4). In comparison with ENP, EPFA have slight larger particle size. This may be due to surface modification by conjugation of folic acid.

Figure 4. Transmission electron microscopy images of ENP (A) and EPFA (B).

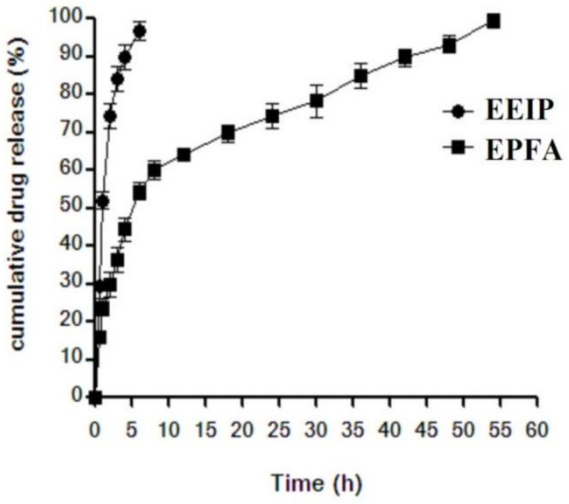


***In vitro* release study**

In vitro release of the EEIP from solution of EPFA was investigated by diffusion bag technique. As EPFA was developed aiming at its intravenous administration, the release studies was conducted phosphate buffer saline (pH 7.4) at 37°C. Figure 5 revealed that EEIP could freely diffuse in its solution form causing 96.79% drug release within 6h. However, EEIP release from polymeric nanoparticles showed a biphasic pattern with initial burst release (23.78 % respectively) within the first 1 h followed by sustained release up to 54 h for EPFA.

Erosion, diffusion and degradation are three basic mechanisms contributed for the release of a loaded drug from polymeric nanoparticles. For developed system any or all three mechanisms may be involved in drug release. During the *in vitro* release study, the initial burst release may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a controlled release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Figure 5 the release profile of EEIP from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by a initial rapid release followed by a slower continuous release phase over 54 h.

Figure 5. *in vitro* release of pure EEIP and EEIP from EPFA (B).



***In vitro* anticancer activity**

The *in vitro* anticancer activity of EPFA was investigated and compared with EEIP against human breast cancer MCF-7 cells using *in vitro* SRB assay.

The results illustrated in Table 3, Figure 6 indicated that EPFA displayed better anticancer activity than EEIP. The total growth inhibition concentration of EPFA (17.0 µg/mL) was approximately 43.34% (30.10 µg/mL) less than the EEIP in solution respectively.

The enhanced anticancer efficacy may be attributed to greater cellular uptake of EPFA via phagocytosis or the fusion process which resulted in the enhanced permeability of the cell membrane to EEIP that allows sufficient drug concentration inside the cells. Therefore, EPFA might have served as a potential nanocarrier to improve *in vitro* anticancer activity of EEIP. The lower anticancer activity of free EEIP in solution may be due to its efflux by P-glycoprotein pumps. The GI50 values for EEIP and EPFA were observed less than 10 µg/mL indicates potent anticancer nature.

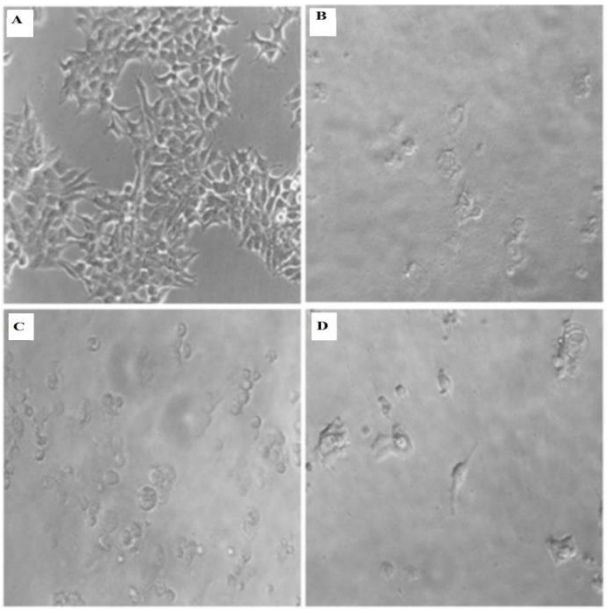
Table 3. TGI and GI_{50} values of EEIP, EPFA and ADR on MCF-7 breast cancer cell line.

Samples	MCF-7 cell line TGI (µg/mL)	MCF-7 cell line GI_{50} (µg/mL)
EEIP	30.10	< 10
EPFA	17.0	< 10
ADR	< 10	< 10

Values were presented as mean ± SD (n = 3).

TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produce 50% inhibition of cells, MCF 7- cell line.

Figure 6. *In vitro* cytotoxicity study on MCF-7 Cell lines (A) MCF-7 control (B) Positive control ADR (C) EEIP (D) EPFA Treated



Hemolysis study

For *in vivo* application it is necessary to evaluate the biosafety by hemolysis study. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate for up to 1.5 mg/mL concentration of EEIP and EPFA were 2.8 and 2.6 % respectively. The acceptable hemolysis rate (less than 3%) [21] Shown denotes formulations having non hemolytic property up to 1.5 mg/mL and may be considered as biosafe for internal use.

***In vivo* anticancer study**

Effect on Tumor Growth, Hematological Parameters and Biochemical Parameters

In the DLA tumor control group, the average life span of animal was found to be 48%. Treatment with EEIP at a dose of 100,200,400 mg/kg increase in body weight and life span was observed as shown in table 4 whereas EPFA nanoparticle formulation at a dose of 100,200,400 mg/kg body weight increase the life span to 93.5%, 94% and 94.5% respectively. In case of EPFA percentage increase in life span was observed that may be because of increased solubility of EEIP when formulated as nanoparticles system. These values were significantly different from normal control (G_1) at $P < 0.01$ and cancer control (G_2) at $P < 0.01$. However the average life span of 5-FU treatment was found to be 96%, indicating its potent antitumor nature. The antitumor nature of EPFA nanoparticle formulation at a dose of 100,200,400 mg/kg was evidenced by the significant reduction in percent increase in body weight of animal when compared to DLA tumor bearing mice. It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in EPFA nanoparticle formulation at a dose of 100,200,400 mg/kg treatments when compared to the DLA tumor control. (Table 4 & 5). In case of EPFA more reduction in packed cell volume and viable Tumor cell count was observed that may be due to increased solubility of EEIP. Due to nanoparticles formulation, drug availability might have increased on site of action because of achieving EPR effect and greater cellular uptake due to reduction in particle size.

Table 4. Effect of EPFA on the life span, body weight and cancer cell count of tumor induced mice

Treatment Groups	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G1	6	>>30 days	2.22±0.68	-
G2	6	48%	9.44±1.86^{a**}	2.75±0.80^{a**}
G3	6	96%	5.66±0.42^{b**}	1.30±0.22^{b**}
G4	6	88%	5.45±0.32^{b**}	1.42±0.30^{b**}
G5	6	89%	5.36±0.30^{b**}	1.40±0.28^{b**}
G6	6	90%	5.30±0.28^{b**}	1.38±0.24^{b**}
G7	6	93.5%	4.94±0.14^{b**}	1.32±0.12^{b**}
G8	6	94%	4.92±0.10^{b**}	1.31±0.08^{b**}
G9	6	94.5%	4.90±0.08^{b**}	1.30±0.05^{b**}

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (EPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

As shown in (Table 5) RBC, Hb, platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with EPFA nanoparticle formulation at a dose of 100,200,400 mg/kg significantly increases the Hb content, RBC, platelets and significantly decreased the WBC count to about normal level. From the results it can be concluded that the EPFA nanoparticle formulation showed comparatively better effects on hematological parameters that showed the developed system is biocompatible with less hemolysis.

Table 5. Effect of EPFA on Hematological parameters

Treatment Groups	Total WBC Cells /mlx10^3	RBC Count millions/mm^3	Hb gm/dl	PCV %	Platelets Lakhs/ mm^3
G1	10.35 ±1.05	4.55±1.95	12.90 ±1.95	14.25±2.44	3.60±0.95

G2	15.30 ±2.60[a**]	2.70±0.98[a**]	6.80 ±0.95	38.36±3.35[a**]	1.70±0.42[a**]
G3	12.30 ±1.34[b**]	4.05±1.62[b**]	11.90±1.48[b**]	16.40±1.40[b**]	2.94±0.50[b**]
G4	12.12±1.26[b**]	4.06±1.50[b**]	12.22±1.52[b**]	17.30±2.36[b**]	3.30±0.65[b**]
G5	12.05±1.22[b**]	4.08±1.60[b**]	12.25±1.55[b**]	17.24±2.30[b**]	3.36±0.68[b**]
G6	11.85±1.18[b**]	4.12±1.65[b**]	12.35±1.60[b**]	17.20±2.26[b**]	3.40±0.70[b**]
G7	11.48±0.95[b**]	4.35±1.89[b**]	12.55±1.72[b**]	16.64±1.88[b**]	3.58±0.85[b**]
G8	11.44±0.90[b**]	4.38±1.92[b**]	12.58±1.75[b**]	16.60±1.84[b**]	3.62±0.88[b**]
G9	11.40±0.88[b**]	4.40±1.95[b**]	12.62±1.78[b**]	16.56±1.80[b**]	3.65±0.92[b**]

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (EPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

The inoculation of DLA cells caused significant increase in the level of total Cholesterol, Aspartate amino Transferase, Alanine amino Transferase and Alkaline Phosphatase in the tumor control animals(G2), when compared to the normal group. The treatment with EPFA nanoparticle formulation at a dose of 100,200,400 mg/kg body weight reversed these changes towards the normal level (Table 6).

Table 6. Effect of EPFA on serum Enzymes and lipid proteins

Treatment Groups	**Cholesterol (mg/dl)**	**TGL (mg /dl)**	**AST (U/L)**	**ALT (U/L)**	**ALP (U/L)**
G1	108.85±3.05	136.85±2.55	36.40 ±1.65	31.28 ±1.45	132.28 ±2.08
G2	146.95±4.34[a**]	220.28±4.40[a**]	78.6±2.94[a**]	62.32±2.60[a**]	265.30±4.35[a**]
G3	126.30±3.84[b**]	169.15±2.65[b**]	44.40 ±1.72[b**]	34.52±1.70[b**]	154.45±2.40[b**]
G4	117.26±3.42[b*]	160.08±2.55[b*]	42.44 ±2.30[b**]	35.28±1.5	162.45±2.22[b*]

	*	*		5[b**]	*
G5	115.18±3.38[b*] *	156.25±2.50[b*] *	41.60 ±2.20[b**]	34.90±1.42[b**]	160.48±2.18[b*] *
G6	113.36±3.26[b*] *	153.30±2.46[b*] *	40.90 ±2.16[b**]	34.80±1.38[b**]	158.45±2.15[b*] *
G7	110.05±3.02[b*] *	143.65±2.26[b*] *	39.42 ±1.95[b**]	33.35±0.92[b**]	151.30±1.95[b*] *
G8	109.12±2.95[b*] *	142.62±2.20[b*] *	39.32 ±1.90[b**]	33.22±0.85[b**]	150.34±1.88[b*] *
G9	109.20±2.90[b*] *	142.25±2.18[b*] *	39.22 ±1.85[b**]	33.05±0.80[b**]	150.30±1.85[b*] *

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (EPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Stability studies

EPFA were subjected to stability study at 40 ± 2°C/75 ± 5% RH. No significant difference in EE and PS were observed over the period of three months as compared to initial values of freshly prepared nanoparticle formulations. *($p > 0.05$).*

Table 8. Accelerated stability study of EPFA for 3 months

Time (Months)	PS (nm)	Percentage of EE
	EPFA	EPFA
Initial	199±3	74.16±1.96
1	201±3	73.62±0.88
2	204±3	73.02±1.54
3	204±3	72.41±1.28

Values were presented as mean ± SD (n = 3). Data was analyzed by one way ANOVA followed by Dunnett's multiple comparison test with $p < 0.05$.

Conclusion

We have investigated folic acid conjugated nanoparticulate formulation composed of PLGA and PVA as an injectable nano carrier for the delivery of a natural anticancer bioactive, EEIP. The developed formulation displayed smaller particle size, excellent encapsulation efficiency and control release with better stability. EPFA demonstrated increase in anticancer efficacy as compared to EEIP. EPFA demonstrated higher *in vitro* as well as *in vivo* anticancer activity in human breast cancer MCF-7 cells than its free form and synergized anticancer effect which may leads to reduction in dose as well as cost. Developed formulation can be considered as an alternative dosage form for EEIP to improve the biopharmaceutical properties and to enhance therapeutic efficacy in cancer chemotherapy.

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PART 4

Part 4

Formulation development of Poly (ε-caprolactone) nanoparticles for improved therapeutic efficacy of Caffeic acid phenethyl ester as a nano medicine

Introduction

Most active chemical components of honeybee propolis is Caffeic acid phenethyl ester (CAPE), which structurally polyphenolics compound [1] It is also reported that CAPE has no potentially harmful effects on normal cells but CAPE has been shown to inhibit the growth of different types of transformed cells [2, 3] CAPE has strong antitumor activity in cancer cells due to the inhibiting potential of the nuclear factor-kappa B (NF-κB) [4, 5]. CAPE gets hydrolyzed in plasma enzymes by an esterase which leads to rapid clearance and short half-life [6]. The overall effect gets results in poor bioavailability and poor biological performances [7].

The drug dissolution, absorption and bioavailability are directly depending upon solubility factor. The potential effects of drugs get reduced due to its low solubility. Various approaches are reported in order to increase drug solubility which includes solid dispersions, cyclodextrin complex formation or some chemical modifications. There are various limitations associated with these approaches [8]. Polymeric nanoparticle system is one of the approaches established to overcome the limitations. The biodegradable polymeric nanoparticles have been investigated especially for achieving increased drug solubility, drug targeting to cancer cells due to their small particle size (10–1000 nm) by enhance permeability and retention effect, prolonging the biological activity by extending blood circulating time, and increasing drug concentration and residence time of drug at specific site of body [9,10].

Poly (ε-caprolactone) (PCL) is a biodegradable polymer most commonly used for pharmaceutical applications [11, 12, 13]. PCL has various advantages such as biocompatibility, biodegradation and applicability in formulation development [14]. PCL has been used extensively for developing nanoparticulate drug delivery systems. PCL has various advantageous properties such as high hydrophobicity and permeability and its biodegradation end product 6-hydroxycaproic acid is neutral, which does not disturb the pH balance [15]. It has been reported that the due to electrostatic interactions positively charged carriers non-specifically interact with a non target cells and negatively charged components of blood [16, 17]. For the tumour targeting, one of the approach is making the carrier particle surface negatively charge with hydrophilic poloxamers and poloxamines (Pluronic®) for reduction of such type of interactions [18].

Quality by design (QbD) approach helps researchers to understand the effects of critical variables in formulation and process on the product quality. Design of Experiment (DoE) is a key factor involved in QbD. Nanoparticle formulations can be developed using DoE approach to identify critical process parameters, reduce number of experiments, and study the interactions between formulation variables. Factorial design method used to optimize the nanoparticle formulation. [8, 19]

Some nanotechnology based attempts have been reported to overcome these limitations for CAPE as therapeutic agent including anticancer drug delivery system. However these studies have not attempted effects of formulation variables, detail characterization of formulations and in vitro and in vivo cytotoxicity performance [1, 20, 21, 22]. Prompted by the above facts, the present study has been designed to develop Caffeic acid phenethyl ester (CAPE) loaded PCL nanoparticles (denoted as CPL) to solve issue of poor water solubility of CAPE, to achieve controlled drug release and improved *in vitro* anticancer efficacy.

Materials and methods

Materials

CAPE (> 98%), Pluronic® F-68, PCL (poly-ε-caprolactone, mw 65,000 Da), and dialysis bag with a 12,000 molecular weight cut off, were purchased from Sigma-Aldrich Chemical Private Ltd (Bangalore, India). Acetone GR grade, sodium hydroxide and potassium dihydrogen phosphate were purchased from Merck India. All other chemical reagents used were of pharmaceutical grade.

Preparation of CAPE loaded polymeric nanoparticles

Briefly, CAPE was dissolved in 3 ml of acetone containing PCL polymer and Pluronic® F-68 (Table 1). The organic solution was injected into 10 ml distilled water at a rate of 10 ml/min under magnetic stirring at 2000 rpm for 1 h at 40°C. The resultant aqueous nanoparticle suspension was filtered through 0.45 μ filter to remove copolymer aggregates. The concentrations of PCL and Pluronic® F-68 (PF 68) were also optimized and selected to obtain stable suspension. The nanoparticle suspension prepared without Pluronic® was unstable because of aggregation of polymeric particles [9].

Design of experiments DoE approach

A DoE approach was used to determine the optimized nanoparticle formulation. After collecting the nanoparticles, PS and EE values were measured. For the statistical design, Design Expert® Version 10.0 was used and PS and EE were selected as dependent variables while the amount of polymer (PCL) and amount of surfactant (PF 68) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of each system were performed.

Particle size analysis, Drug content and Encapsulation Efficiency (EE)

The PS of developed formulations was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The PS measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average PS was determined, expressed in terms of d (0.9) μm.

The concentration of active ingredients (DC and EE) in the polymeric nanoparticle formulation was measured by high performance liquid chromatography method (HPLC, Jasco UV 2057, Japan). A mobile phase consisting of methanol and water (80:20 v/v) at flow

rate of 1.0 ml/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 µ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 µL, and the detection wavelength was set at 331 nm. Temperature was maintained at $25^0 \pm 2^0$ C. The nanoparticle solution was suitably diluted with alcohol prior to determination.

The percent DC was calculated according to the equation:
DC (%) = (Amount of CAPE in nanoparticle/ Amount of CAPE and polymer) × 100 (1)
EE was determined by separating non-encapsulated active ingredients from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4^0C. The sediment nanoparticles were disrupted with acetone to release the entrapped CAPE; suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method, (Jasco UV 2057, Japan)
The percent EE was calculated using Equation (2).
EE (%) = Amount of CAPE entrapped in the vesicle/ Initial amount of the CAPE added × 100. (2)

Optimization by 3^2 factorial design

Preliminary experiments helped in understanding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PCL (X1) and PF 68 (X2) concentration were identified as factors crucial in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was adopted to optimize the nanoparticles composition while studying the effect of X1 and X2 (independent variables) on PS and EE (two dependent variables or responses) by obtaining a 3D response surface plot.

Zeta potential
The zeta potential was measured with the laser Doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments) at a temperature of 25°C.

Differential scanning calorimetry
The differential scanning calorimetry (DSC) thermograms of CAPE, PCL and CPL (vacuum-evaporated samples) were obtained using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples were (5 mg) heated in hermetically sealed aluminium pan with a heating rate of 10°C/min under a nitrogen atmosphere (flow rate 50 ml/min).

Fourier transform-infrared spectroscopy (FTIR)
FTIR spectra of CAPE, PCL and nanoparticle formulations were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan) upon mixing in dry KBr. Each sample was scanned in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of nanoparticle suspensions was studied using transmission electron microscopy (TEM). A drop of CPL was applied to a carbon film-covered copper grid. Most

of the suspension was blotted from the grid with filter paper to form a thin film specimen. The unstained sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV and a magnification of 50000X.

In vitro drug release study

Polymeric nanoparticle formulations (each 5 ml) were placed into a dialysis bag (cut off 12,000D).The bag was immersed into a beaker containing phosphate buffer (pH 7.4) at 37 ± 0.5 °C with continuous magnetic stirring at 100 rpm. At predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36 and 42 h) the sample (5 ml) was withdrawn maintaining sink condition. The samples were analyzed for CAPE content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of CAPE was plotted against time. Pure CAPE release was analyzed in a similar manner [23].

In vitro cytotoxicity studies

Cytotoxic activity of CAPE and CPL was evaluated on human breast cancer MCF-7 and human colon cancer HT-9 cell line using sulforhodamine B (SRB) assay. The study performed as per the procedure described by Bothiraja et al, 2013 at Tata Memorial Center, Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Formulations were diluted in distilled water so as to obtain CAPE in the concentration ranges of 10, 20, 40 and 80µg/ml which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^3 cells per well in 96-well plates using in-situ fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37ºC with 100% relative humidity (RH), the growth medium was replaced with 100 µL of fresh medium containing various concentrations (10-80 µg/mL) of CAPE and CPL. The culture media without any drug formulation was used as a control. After 48 h incubation, assay was terminated by adding 50 µL of the cold TCA and incubated for 60 min at 4ºC. The media was removed and washed with sterile PBS and dried. 50 µL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation.

Cell growth (%) = (Average absorbance of the test well / Average absorbance of the control wells) ×100 [24].

Haemolysis study

The hemolytic effects of the CAPE and CPL were determined using the method described by love et al. briefly, fresh 10 ml blood collected from healthy donors and fibrinogen was

removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. after removal of supernatant the precipitate diluted with aqueous NaCl solution (1:50 v/v) to get 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number represented a set of three tubes. To all of the tubes 2.5 mL of the red blood cell suspension was added. The tubes labeled 1 were diluted with 2.5 mL of distilled water as the hemolysis control (100% hemolysis) and the tubes labeled 2 were diluted with 2.5 mL of 0.9% isotonic NaCl solution as the non hemolysis control (0% hemolysis). The remaining tubes were diluted with CAPE and CPL samples corresponding to concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/mL of formulations and volume made up to 5 mL with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer [25]

Stability study

CPL was subjected to accelerated stability studies for three months as per ICH guidelines at a temperature of 25 ± 2^0 C and 60% RH. Optimized nanoparticles were analyzed for the change in PS and EE.

Results and discussion

Nanomedicine based drug delivery systems has proven for improving the efficacy and safety of various drugs over the past few decades [26-28]. In this study, CAPE loaded polymeric nanoparticles have been developed and investigated as oral nanocarriers in order to improve its solubility, *in vitro* anticancer efficacy. The effect of the CAPE loaded polymeric nanoparticles composition on PS and EE was studied by design of experiments (DoE) approach.

Effect of polymer concentration on PS and EE

Initially effect of CAPE: PCL at different ratios (1:2.5/5/7.5/10) on particle size and encapsulation efficiency was studied. At 1:5 ratio minimum PS and maximum EE was observed. Further CAPE: PCL at ratios of (1:3.75/5/6.25) was studied. At all ratio results obtained was satisfactory to our requirement. Further detail studies considering surfactant concentrations, dependant and independent variables were done for final optimization of formulation batch.

PS, DC & EE

The mean CPL nanoparticles size was in the range of 187 $\pm$ 2 to 220 $\pm$ 5 and was strongly affected by the selected variables. The DC and EE were in the range of 90.13–96.80 % and 64.37–74.80 %, respectively for CPL. (Table 1) A good fit (r^2 for PS = 0.9284 and EE = 0.8134 for CNP) was observed for the PS and EE the independent variables.

Drug delivery to specific sites of the body is influenced by size of the nanoparticles; smaller particles may tend to minimize the particle uptake by non targeted cells, including their premature clearance by the mononuclear phagocytic system [29].

Table1. The effect of various formulations (with different amounts of surfactant and polymer) on PS and EE by 3^2 factorial design with coded levels and actual amount values of variables.

Batches CPL	Coded levels (X1, X_2)	Amt. of PCL (mg; X_1)	Amt of PF 68 (mg; X_2)	Drug Content (%)	Nanoparticle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)
F1	+1, +1	62.50	100.00	90.15± 0.91	220± 2	64.37± 1.20
F2	+1, 0	62.50	75.00	90.13± 0.76	209± 2	67.73± 1.45
F3	+1,-1	62.50	50.00	90.83± 0.91	209± 3	67.83± 1.45
F4	0, +1	50.00	100.00	95.42± 0.76	199± 3	69.42± 1.80
F5	0, 0	50.00	75.00	95.61± 0.48	197± 3	70.61± 1.80
F6	0, -1	50.00	50.00	96.80± 0.48	191± 2	74.80± 1.45
F7	-1, +1	37.50	100.00	95.62± 0.91	190± 3	70.62± 1.45
F8	-1, 0	37.50	75.00	95.12± 0.76	188± 2	68.62± 1.20
F9	-1, -1	37.50	50.00	94.66± 0.91	187± 2	67.66± 1.45

Optimization of CPL nanoparticles by 3^2 factorial design

PS and EE are the major important factors considered in formulation development of nanoparticles. During the preliminary study, assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of polymer and surfactant (Table 1). CAPE content was kept constant. As per 3^2 factorial design, nine different batches were prepared for each system. The responses of these batches are shown in Table 1, Table 2. The data obtained was subjected to multiple regression analysis using Design Expert® Version 10.0. The results of multiple regression analysis of the obtained data are summarized in Table 2.

From the factorial design study of CPL (Table 2), we observed that positive coefficients of the main terms X1 and X2 indicated a favorable effect on the mean particle size and EE with the PCL and PF68. The PCL had a linear effect on the PS and curvilinear effect on EE while PF 68 had linear effects on both PS and EE as seen in surface plot (Figure 1).

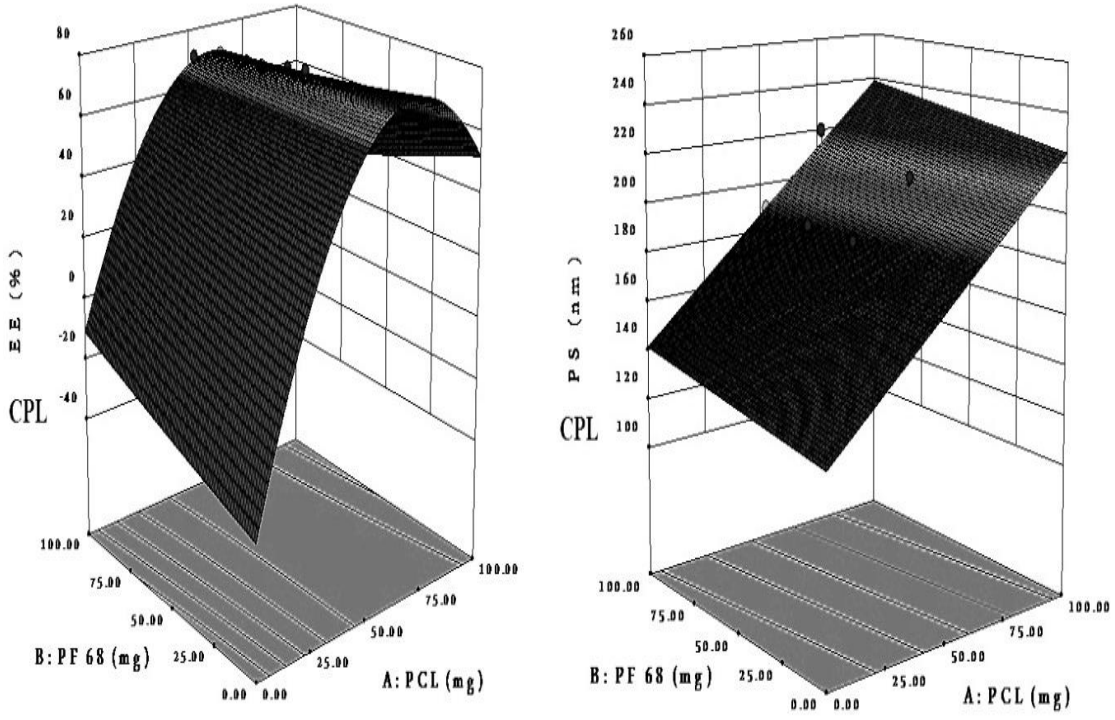
Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.852 was obtained with F6 for CPL so F6 batch was selected as an optimized formulations and further evaluated for various parameters.

Table 2. The results of multiple regression analysis of the obtained data.

Sr. no	Final Equation in Terms of Coded Factors		
	EE	PS	
1	+68.38	+183.06	=
2	+32.21	+48.67	* X1
3	+0.73	+7.33	* X2
4	-12.84		* X1X2
5	-60.88		* $X1^2$
6	+0.52		* $X2^2$

Number	PLGA	PVA	EE	PS	Desirability
CPL	55.286	50.000	72.051	191.121	0.852

Figure 1. Response surface plot showing effects of amount of PCL and PF 68 (independent variables) on dependent variables PS and EE for CPL



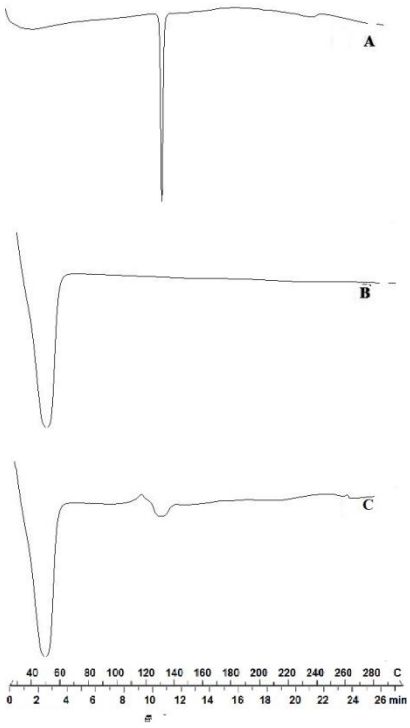
Zeta potential

Zeta potential is essential parameter gives information about surface charges surface charges which has direct influence on colloidal stability and interaction with physiological body cells. The CPL showed negative zeta potential (–23.01 ± 0.45 mV) which may be due to presence of PF 68 on surface. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability and blood clearance.

Differential scanning calorimetry

Study was performed for the free CAPE, PCL and CPL (Figure 2) in order to determine the molecular state of the CAPE. A sharp melting transition of free CAPE was observed at 127.48 °C with \H 38.01 J/g shows transit crystallinity. In CPL thermogram, the free CAPE peak was disappeared indicating molecular dispersion of CAPE inside polymeric nanoparticles.

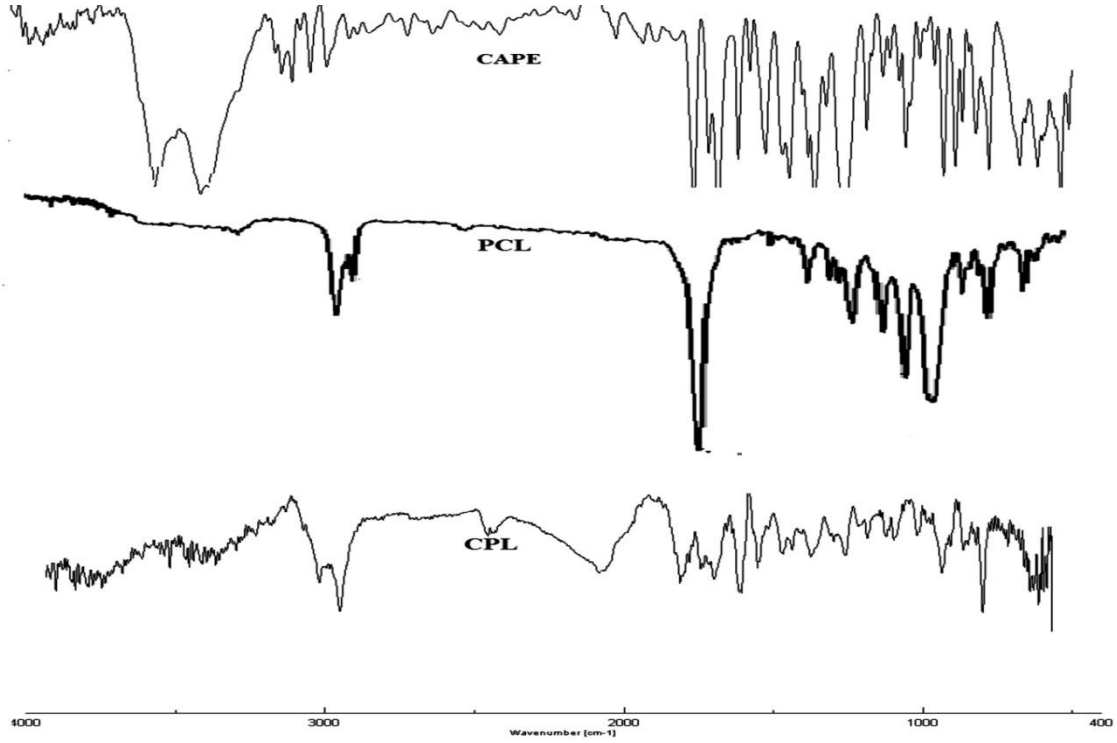
Figure 2. Comparative DSC thermo grams of CAPE (A), PCL (B), CPL (C)



Fourier transform-infrared spectroscopy (FTIR)

Figure 3 shows FTIR spectra of pure CAPE (A), PCL (B) and CPL (C). It can be seen from the FT-IR spectrum of free CAPE that the bands obtained at 3482 cm^{-1} and 3333 cm^{-1} were assigned to –OH stretching. Band obtained at 3061.44 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1683 cm^{-1}, 1602 cm^{-1}, and 1184 cm^{-1} were also attributed C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PCL the bands at 2940 cm^{-1} and 2860 cm^{-1} were C–H hydroxyl groups asymmetric stretching and C-H hydroxyl groups symmetric stretching respectively. 1722 cm^{-1} is assigned to C=O stretching vibrations of the ester carbonyl group. The absorption at 1238 cm^{-1} is assigned to C-O-C asymmetric stretching and 1160 cm^{-1} is C-O-C symmetric stretching. However, in the FTIR spectra of CPL, the major peaks of CAPE at 3482 cm^{-1}, 3333cm^{-1}, and 1602cm^{-1} were significantly decreased and the presence of these characteristic peaks is a confirmation of CAPE encapsulation on PCL nanoparticles successfully.

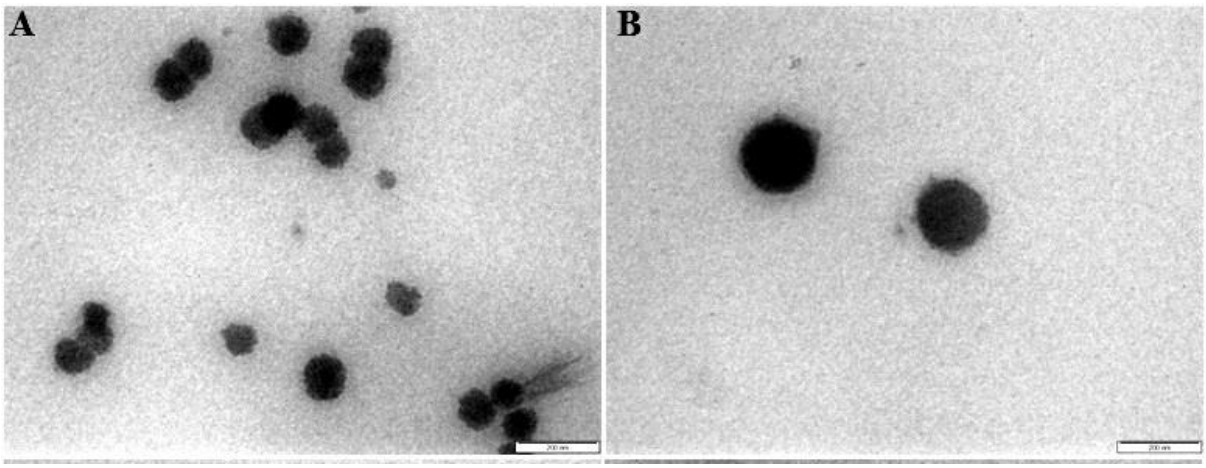
Figure 3. Comparative FTIR spectra of pure CAPE, PCL, and CPL



Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of the CPL was assessed using transmission electron microscope (TEM) from which it can be seen that the nanoparticles were freely dispersed and spherical in shape (Figure 4). Little bigger size nanoparticles were observed in TEM than that tested by laser diffraction technique. Because of low melting points of PF 68 (~55 °C) and PCL (~60 °C), nanoparticles may undergoes the melting due to high energy electron beam in TEM. Melting might cause expansion to certain level and nanoparticles seem bigger in TEM.

Figure 4. Transmission electron microscopy images of CPL (A&B)

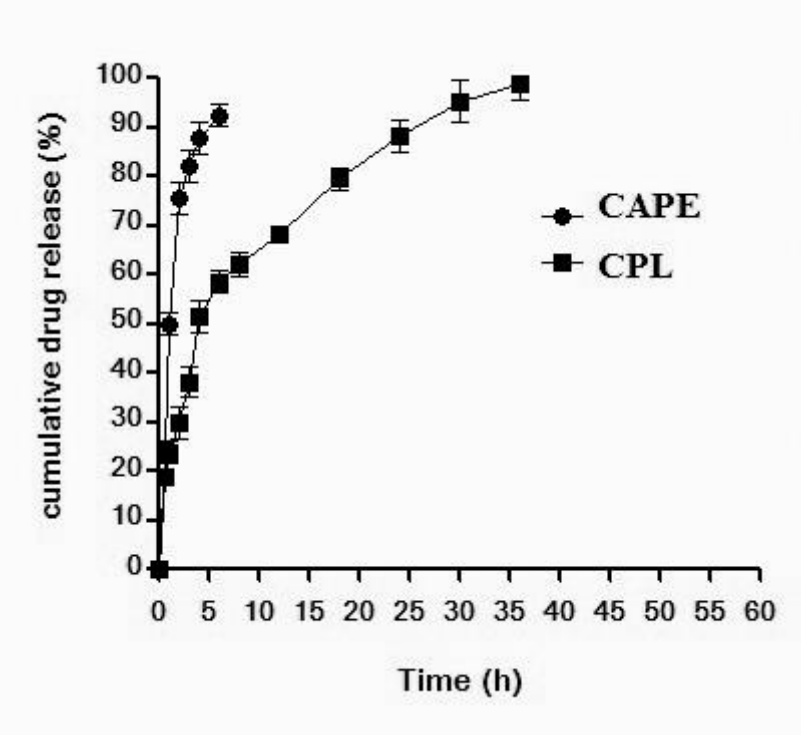


***In vitro* release study**

In vitro release of the CAPE from solution of CPL was investigated by diffusion bag technique. As CPL was developed aiming at its intravenous administration, the release studies were conducted in phosphate buffer saline (pH 7.4) at 37°C. Figure 5 revealed that CAPE could freely diffuse in its solution form causing 92.39% drug release within 6 h. However, CAPE release from polymeric nanoparticles showed a biphasic pattern with initial burst release (19.00%) within the first 1 h followed by sustained release up to 36 h for CPL.

Erosion, diffusion and degradation are three basic mechanisms contributed for the release of a loaded drug from polymeric nanoparticles. For developed system any or all three mechanisms may be involved in drug release. During the *in vitro* release study, the initial burst release may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a controlled release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Figure 5 the release profile of CAPE from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by a initial rapid release followed by a slower continuous release phase over 36 h.

Figure 5. *in vitro* release of pure CAPE and CAPE from CPL



***In vitro* anticancer activity**

The *in vitro* anticancer activity of CPL was investigated and compared with CAPE against human breast cancer MCF-7 and HT-29 cells using *in vitro* SRB assay.

The results illustrated in Table 3, Figure 6 indicated that CPL displayed superior anticancer activity than CAPE on breast cancer MCF-7 cells. The total growth inhibition concentration of CPL was observed 27.20 µg/ml while for CAPE it was 46.00 µg/ml.

In case of colon cancer HT-29 cells lines Table 3, Figure 7 indicated that CPL displayed superior anticancer activity than CAPE. The total growth inhibition concentration of CPL was observed 36.00 µg/ml and for CAPE 47.20% µg/ml.

The enhanced anticancer efficacy may be attributed to greater cellular uptake of CPL via phagocytosis or the fusion process which resulted in the enhanced permeability of the cell membrane to CAPE that allows sufficient drug concentration inside the cells. Therefore, CPL might serve as a potential nanocarrier to improve *in vitro* anticancer activity of CAPE. The lower anticancer activity of free CAPE in solution may be due to its efflux by P-glycoprotein pumps. The GI50 values for CAPE and CPL were observed less than 10 µg/ml indicates potent anticancer nature on both cell lines.

Table 3. TGI and GI_{50} values of CAPE, CPL and ADR on MCF-7 breast cancer and HT-29 colon cancer cell line.

Samples	**MCF-7 cell line TGI (µg/mL)**	**MCF-7 cell line GI_{50} (µg/mL)**	**HT-29 cell line TGI (µg/mL)**	**HT- 29 cell line GI_{50} (µg/mL)**
CAPE	46.00	12.1	47.20	20.10
CPL	27.20	< 10	36.00	12.00
ADR	< 10	< 10	< 10	< 10

Values were presented as mean ± SD (n = 3).

TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produces 50% inhibition of cells, MCF 7 and HT-29 cell line.

Figure 6. *In vitro* cytotoxicity study on MCF-7 Cell lines (A) MCF-7 control (B) Positive control ADR (C) CAPE (D) CPL Treated

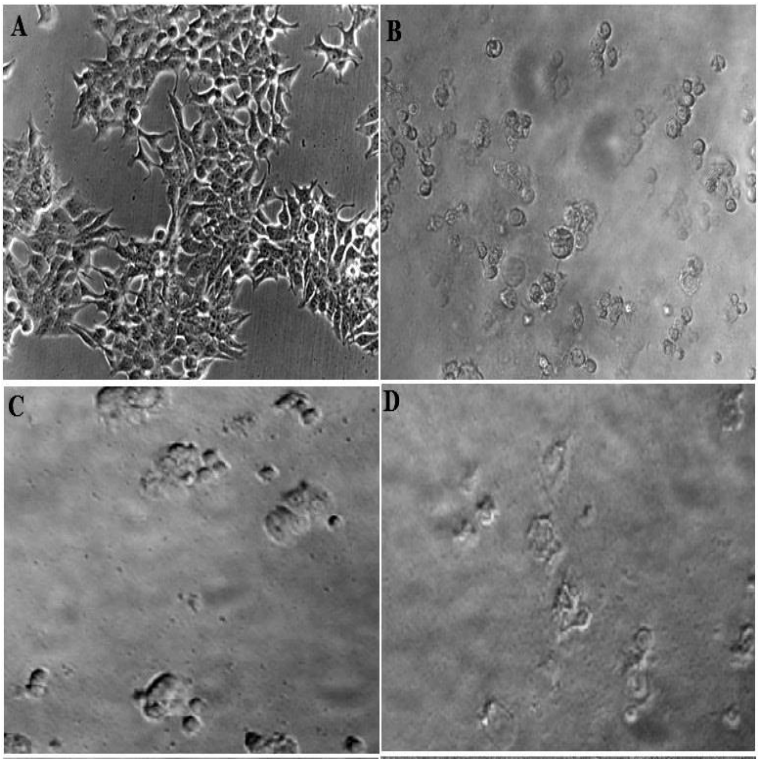
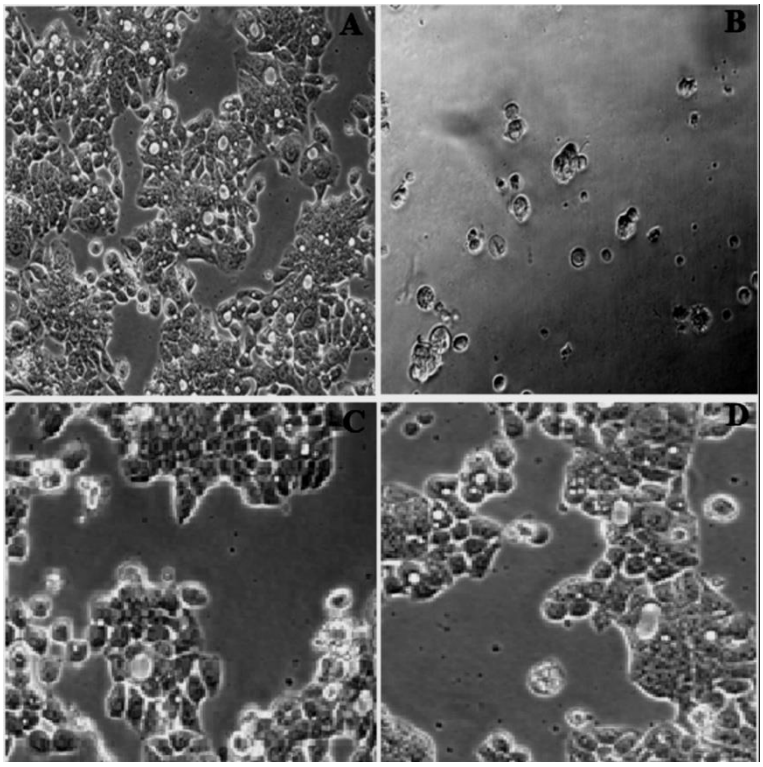


Figure 7. *In vitro* cytotoxicity study on HT-29 Cell lines (A) MCF-7 control (B) Positive control ADR (C) CAPE (D) CPL Treated



Hemolysis study

For *in vivo* application it is necessary to evaluate the biosafety by hemolysis study. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate for up to 1.5 mg/mL concentration of CAPE and CPL were 2.5 and 2.6, % respectively. The acceptable hemolysis rate (less than 3%) [21] Shown denotes formulations having non hemolytic property up to 1.5 mg/mL and may be considered as biosafe for internal use.

Stability studies

CPL was subjected to stability study at 40 ± 2°C/75 ± 5% RH. No significant difference in EE and PS were observed over the period of three months as compared to initial values of freshly prepared nanoparticle formulations. ($p > 0.05$).

Table 4. Accelerated stability study of CPL for 3 months

Time (Months)	PS (nm)	Percentage of EE
	CPL	CPL
Initial	191±3	74.80±1.18
1	193±2	74.25±0.94
2	193±2	73.82±1.08
3	194±3	73.20±0.94

Values were presented as mean ± SD (n = 3). Data was analyzed by one way ANOVA followed by Dunnett's multiple comparison test with $p < 0.05$.

Conclusion

We have investigated nanoparticulate formulation composed of PCL and PF-68 as an injectable nano carrier for the delivery of a lipophilic anticancer bioactive, CAPE. The developed formulation displayed smaller particle size, excellent encapsulation efficiency and control release with better stability. CPL demonstrated increase in anticancer efficacy as compared to CAPE. CPL demonstrated higher *in vitro* anticancer activity in human breast cancer MCF-7 cells and human colon cancer HT-29 than its free form, which may leads to reduction in dose as well as cost. Developed formulation can be considered as an alternative dosage form for CAPE to improve the biopharmaceutical properties and to enhance therapeutic efficacy in cancer chemotherapy.

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PART 5

Part 5

Caffeic Acid Phenethyl Ester Loaded Folic Acid Conjugated Bioactive Nanoparticles: *in-Vitro* and *in-Vivo* Anticancer Study

Introduction

Caffeic acid phenethyl ester (CAPE), the active chemical constituent of honeybee propolis is structurally polyphenolic compound [1, 2]. CAPE possess antitumor activity in different cancer cells by various mechanisms which includes induction of the activity of p21protein, p38 MAPK, p53 and JNK kinase and inhibition of the nuclear factor-kappa B (NF-□B), increased activity of caspase-3 or caspase-7 [3, 4, 5]. It is also reported that CAPE has no harmful effects on normal cells [6] but CAPE has been shown to inhibit the growth of different types of transformed cells [7]. Although CAPE has beneficial anticancer activity on human cancer cell lines but it has some limitations such as it is highly lipophilic in nature and poorly soluble in water. It gets hydrolyzed in plasma enzymes by an esterase [8] which results in rapid clearance and short half-life. The overall effect leads to low bioavailability and poor biological activity [9].

The drug dissolution, absorption and bioavailability are directly depending upon solubility factor. The potential effects of drugs get reduced due to its low solubility. Various approaches are reported in order to increase drug solubility which includes solid dispersions, cyclodextrin complex formation or some chemical modifications. There are various limitations associated with these approaches [10]. Polymeric nanoparticle system is one of the approaches established to overcome these limitations. The biodegradable polymeric nanoparticles have been investigated especially for achieving increased drug solubility, drug targeting to cancer cells due to their small particle size (10–1000 nm) by enhanced permeability and retention effect (EPR), prolonging the biological activity by extending blood circulating time, and increasing drug concentration and residence time of drug at specific site of body [11].

Although a number of different biodegradable polymers have been investigated for formulating nanoparticles, Poly (D, L-lactide-co-glycolide) (PLGA) have been widely used for controlled drug delivery systems as a carrier for various therapeutic agents because of its high biocompatibility, biodegradability and low toxicity [12, 13]. Further, Folic acid, a water soluble vitamin which is oxidized form of folate is used as a ligand due to its efficient internalization via folate receptor mediated endocytosis for tumor targeting. Folic acid is abundantly expressed in various types of human tumors such as breast, ovarian and prostate cancer. But in case of normal tissues it is minimally distributed. Therefore, Folic acid can serve as potential tumor marker and a functional tumor-specific receptor [14, 15, 16].

Most of studies reported on CAPE focus on chemical synthesis and biological evaluation [2]. Some nanotechnology based attempts have been reported to overcome these limitations for CAPE as therapeutic agent including anticancer drug delivery system. However these studies have not attempted effects of formulation variables, detail characterization of formulations and in *vitro* and *in vivo* cytotoxicity performance [17, 18, 19, 20]. Prompted by the above facts, the present study has been designed to develop Caffeic acid phenethyl ester (CAPE)

loaded PLGA– folic acid conjugated polymeric nanoparticles (denoted as CPFA) using Design of Experiment approach to solve issue of poor water solubility of CAPE, achieve sustain drug release and increased *in vitro* and *in vivo* cytotoxicity.

Materials and Methods

Materials

CAPE (> 98%), PLGA with a copolymer ratio of d-l-lactide to glycolide of 50:50 (Mw 40,000–100,000 g/mol), poly(vinyl alcohol) PVA (87–89% hydrolysis degree and molecular mass 12,000–13,000 g/mol, dialysis bag with a 12,000 Da molecular weight cut off, 1,3,Dicyclo-hexyl carbodiimide (DCC), N-hydroxysulfosuccinamide (Sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Bangalore, India. Acetone GR grade, sodium hydroxide, potassium dihydrogen phosphate, dichoromethane (DCM) and ethanol were purchased from Merck India. All other chemical reagents used were of analytical grade.

Preparation of CAPE loaded polymeric nanoparticles

CAPE loaded PLGA nanoparticles (denoted as CNP) were prepared by oil in water (o/w) single emulsion solvent evaporation method described by Song *et al.*, 2008 with minor modifications. Formulation batches were prepared as per composition shown in Table 1. The amount of CAPE (10 mg) kept constant. The organic phases consisted of PLGA dissolved in dichloromethane (DCM), CAPE was dissolved in ethanol. CAPE and PLGA as per factorial design composition shown in Table 1 were mixed and stirred to ensure that all materials were dissolved. This organic solution was emulsified with the 5 mL aqueous solution of PVA as per different proportions shown in Table 1 by sonication (output power 70W, power of 80%, and 2 minutes) using a probe sonicator (U 200 S Control, Ika labrortech staufen, Germany) over an ice bath. The o/w single emulsions were stirred overnight on a magnetic stirrer at 1500 rpm at room temperature for evaporation of organic phase. The resulting particles were collected by centrifugation at 9000x rpm for 40 min (eppendorf Centrifuge 5424 R), washed three times with ultra-pure water to remove excess PVA. Polymeric nanoparticles were separated from the free active ingredients or polymer agglomerate by filtration through a 0.45 μm membrane filter and then lyophilized. Lyophilized nanoparticles were stored at -80^0 C until used. [21]

Effect of polymer concentration particle size (PS) and encapsulation efficiency (EE)

The effect of polymer concentration was studied by studying effects of different CAPE: PLGA ratio upon PS and EE.

Design of experiments DoE approach

A DoE approach was used to determine the optimized nanoparticle formulation. After collecting the nanoparticles, particle size (PS) and encapsulation efficiency (EE) values were measured. For the statistical design, Design Expert® Version 10.0 was used and PS and EE were selected as dependent variables while the amount of polymer (PLGA) and amount of

surfactant (PVA) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of each system were performed.

DC, PS and EE analysis

The PS of developed formulations was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The PS measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average PS was determined, expressed in terms of d (0.9) μm.

The concentration of active ingredients (DC and EE) in the polymeric nanoparticle formulations were measured by HPLC method. A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 mL/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 μ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 μL, and the detection wavelength was set at 331 nm. Temperature was maintained at $25^0 \pm 2^0$ C. The nanoparticle solution was suitably diluted with alcohol prior to determination.

The percent DC was calculated according to the equation:
DC (%) = (Amount of CAPE in nanoparticle/ Amount of CAPE and polymer) × 100 (1)
EE was determined by separating non-encapsulated active ingredients from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4^0C. The sediment nanoparticles were disrupted with acetone to release the entrapped CAPE; suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method, (Jasco UV 2057, Japan)
The percent EE was calculated using Equation (2).
EE (%) = Amount of CAPE entrapped in the vesicle/ Initial amount of the CAPE added × 100. (2)

Optimization by 3^2 factorial design

Preliminary experiments helped in understanding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PLGA (X1) and PVA (X2) concentration were identified as factors crucial in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was adopted to optimize the nanoparticles composition while studying the effect of X1 and X2 (independent variables) on PS and EE (two dependent variables or responses) by obtaining a 3D response surface plot.

Surface conjugation with folic acid

The surface modification was carried out by previously published method described by Das and Sahoo *et al*., 2012 with minor modification. Folic acid was reacted at stoichiometric molar ratio of Folic acid: DCC: NHS = 1:1.2:2 in dimethyl sulphoxide (DMSO) for 6 hrs at 50^0C. N, N- dicyclohexylurea (DCU) formed in reaction was removed by filtration. Formed product was allowed to react over night with ethylene diamine in presence of pyridine which

acts as catalyst. The aminated folate formed was precipitated out by adding excess cold acetonitrile, separated by vacuum filtration. For conjugation of aminated folate onto the surface of optimized CNP, 20 mg of CNP was dispersed in 10 mL of phosphate buffer solution (PBS) (0.02 M, pH 7.4). 250 µl of EDC (2 mg/mL) and NHS (2 mg/mL) each were added to the above suspension followed by agitating for 2 hrs at room temperature using a magnetic stirrer. By ultracentrifugation at 20,000 rpm for 20 min at 4^0C (eppendorf Centrifuge 5424 R), excess of unreacted EDC and NHS were removed and activated nanoparticles were recovered. For final conjugation, activated nanoparticles were dispersed in 4 mL of PBS (0.02 M, pH 7.4) followed by addition of 200 µl of aminated folate solution (1mg/mL in PBS). The solution was again agitated for 2 hrs at room temperature and excess of unconjugated folate was removed by ultracentrifugation and lyophilized for further use. Conjugation was confirmed by Fourier transform-infrared spectroscopy (FTIR) analysis. [22]

Zeta potential

The zeta potential was measured with the laser Doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments, UK) at a temperature of 25^0C.

Differential scanning calorimetry (DSC)

DSC thermograms of CAPE and CPFA (vacuum-evaporated samples) were obtained using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples were (5 mg) heated in hermetically sealed aluminium pan with a heating rate of 10^0C/min under a nitrogen atmosphere (flow rate 50 mL/min).

Fourier transform-infrared spectroscopy (FTIR)

FTIR spectra of CAPE, PLGA, Folic acid and nanoparticle formulations were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan) upon mixing in dry KBr. Each sample was scanned in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of nanoparticle suspensions was studied using transmission electron microscopy (TEM). A drop of CNP and CPFA suspension was applied to a carbon film-covered copper grid. Most of the suspension was blotted from the grid with filter paper to form a thin film specimen. The unstained sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV and a magnification of 50000X.

***In vitro* drug release study**

The *in vitro* release of CAPE and CPFA from the nanoparticle formulations were carried out in phosphate-buffer saline (pH 7.4) using dialysis bag diffusion technique. Polymeric nanoparticle formulations equivalent to 2 mg of CAPE and 2 mg CAPE solution (2 mg/mL in 50% w/w mixture of PEG 400 and water) as control was were placed into a dialysis bag (cut off 12,000D).The bag was immersed into a beaker containing in 50 mL of release medium with continuous magnetic stirring at 100 rpm at 37 $\pm$ 0.5 ^{0}C. At

predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48 and 54 h) the sample (5 mL) was withdrawn maintaining sink condition. The samples were analyzed in triplicate for CAPE content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of CAPE was plotted against time. Pure CAPE release was analyzed in a similar manner [23]

In vitro cytotoxicity studies

Cytotoxic activity of CAPE and CPFA were evaluated on human breast cancer cell line MCF-7 using sulforhodamine B (SRB) assay at Tata Memorial Center, Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Formulations were diluted in distilled water so as to obtain CAPE in the concentration ranges of 10, 20, 40 and 80μg/mL which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^3 cells per well in 96- well plates using in-situ fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37ºC with 100% relative humidity (RH), the growth medium was replaced with 100 μL of fresh medium containing various concentrations (10-80 μg/mL) of CAPE and CPFA. The culture media without any drug formulation was used as a control. After 48 h incubation, assay was terminated by adding 50 μL of the cold TCA and incubated for 60 min at 4ºC. The media was removed and washed with sterile PBS and dried. 50 μL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation.

Cell growth (%) = (Average absorbance of the test well / Average absorbance of the control wells) ×100 [24].

Haemolysis study

The hemolytic effects of the CAPE and CPFA were determined using the method described by love *et al*. briefly, fresh 10 ml blood collected from healthy donors and fibrinogen was removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. after removal of supernatant the precipitate diluted with aqueous NaCl solution (1:50 v/v) to get 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number represented a set of three tubes. To all of the tubes 2.5 mL of the red blood cell suspension was added. The tubes labeled 1 were diluted with 2.5 mL of distilled water as the hemolysis control (100% hemolysis) and the tubes labeled 2 were diluted with 2.5 mL of 0.9% isotonic NaCl solution as the non hemolysis control (0% hemolysis). The remaining tubes were diluted with CAPE and CPFA samples corresponding to concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/mL of formulations and volume made up to 5 mL with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were

centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer [25].

In vivo anticancer study

Selection grouping and acclimatization of laboratory animals and Induction of cancer using DLA cells

The study protocol was approved by the Institutional Animal Ethics Committee (protocol number: CPCSEA/QA/06/2016). Male Swiss albino mice (20-25 gm) were used for the study. They were acclimatized to laboratory environment at (temperature 25 ± 2°C) and 12 h dark /light cycle with diet and water. Dalton's Lymphoma ascites (DLA) cells were supplied by Amla cancer research center, Trissur, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by intraperitoneal (i.p) transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. After cell counts further dilutions were made to total cell count should be $1x10^6$ .Tumor growth in the mice was allowed for minimum seven days before starting treatments.

Treatment Protocol

Animals were divided in to fifteen groups of six each. G2- G15 was injected with DLA cells (1×10^6 cells per mouse) intraperitonially (i.p.).
Group 1(G1) served as the normal control, Group 2 (G2) served as the tumor control. Group 1 and 2 receives normal diet and Water.
Group 3 (G3) served as the positive control and was treated with injection 5-fluorouracil at 20 mg/kg body weight i.p.
Group 4 (G4), Group 5 (G5) and Group 6 (G6) were administered CAPE at a dose of 50 mg/kg, 100 mg/kg and 200 mg/kg body weight i.p. respectively.
Group 7 (G7), Group 8 (G8) and Group 9 (G9) were administered CPFA at a dose of 100 mg/kg , 200 mg/kg and 400 mg/kg body weight i.p respectively.

Evaluation of anticancer activity

Treatment was given after 24 h of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital plexus bleeding and the following parameters were evaluated. Hematological parameters like WBC, RBC count , Hb content, platelet count and packed cell volume; serum enzyme and lipid profile which include total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP); and derived parameter like body weight, life span (%) and cancer cell count were evaluated. The results are expressed as mean ± S.E.M. The evaluation of the data was done using one way ANOVA followed by Newman-Keul's multiple comparison test; $p< 0.01$ implied significance. [26, 27, 28, 29]

Stability study

CPFA were subjected to accelerated stability studies for three months as per ICH guidelines at a temperature of 25 ± 2^0 C and 60% RH. Optimized nanoparticles were analyzed for the change in PS and EE.

Results and Discussion

In this study, CAPE loaded polymeric nanoparticles have been developed and investigated as nano carriers in order to improve solubility, *in vitro* and *in vivo* anticancer efficacy. The effect of the CAPE loaded polymeric nanoparticles composition on PS and EE was studied by design of experiments (DoE) approach.

Effect of polymer concentration PS and EE

Initially effect of CAPE: PLGA at different ratios (1:2.5/5/7.5/10) on particle size and encapsulation efficiency was studied. At 1:5 ratio minimum PS and maximum EE was observed. Further CAPE: PLGA at ratios of (1:3.75/5/6.25) was studied. Results obtained were satisfactory to our requirement. Further detail studies considering surfactant concentrations, dependant and independent variables were done for final optimization of formulation batch.

DC, PS & EE

The mean CNP nanoparticles size was in the range of 170 ± 2 to 195 ± 3 nm and was strongly affected by the selected variables. The DC and EE were in the range of 91.37–99.80 % and 75.66–78.80 % (Table 1). A good fit (r^2 for PS = 0.9845 and EE = 0.9965 for CNP) was observed for the PS and EE the independent variables.

Table1. The effect of various formulations (with different amounts of surfactant and polymer) on PS and EE by 3^2 factorial design with coded levels and actual amount values of variables.

Batches CNP	Coded levels (X1, X_2)	Amt. of PLGA (mg; X_1)	Amt of PVA (mg; X_2)	Drug Content (%)	Nanoparticle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)
F1	+1, +1	62.50	100.00	91.37± 1.23	170± 2	78.80± 1.25
F2	+1, 0	62.50	75.00	93.73± 1.11	174± 2	78.73± 1.11
F3	+1,-1	62.50	50.00	93.83± 2.00	178± 2	78.13± 1.52
F4	0, +1	50.00	100.00	97.42± 1.89	179± 3	77.62± 1.40
F5	0, 0	50.00	75.00	98.61± 1.56	185± 2	77.61± 0.88
F6	0, -1	50.00	50.00	99.80± 0.89	189± 3	77.00± 0.85
F7	-1, +1	37.50	100.00	95.62± 0.99	190± 2	76.62± 1.80
F8	-1, 0	37.50	75.00	95.62± 1.10	194± 3	76.62± 1.00

F9	-1, -1	37.50	50.00	94.66± 1.54	195± 3	75.66± 1.52

Optimization of CNP nanoparticles by 3^2 factorial design

PS and EE are the major important factors considered in formulation development of nanoparticles. During the preliminary study, assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of polymer and surfactant (Table 1). CAPE content was kept constant. As per 3^2 factorial design, nine different batches were prepared. The responses of these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using Design Expert® Version 10.0. The results of multiple regression analysis of the obtained data are summarized in Table 2.

From the factorial design study of CNP (Table 2), it was observed that positive coefficients of the main terms X1 and X2 for EE and negative coefficients of the main terms X1 and X2 for PS indicated a favorable effect on the mean particle size and EE with the PLGA and PVA. The PLGA had a linear effect on both PS and EE while PVA had linear effects on PS and slight curvilinear effect on EE as seen in response surface plot (Figure 1). Smaller particles were obtained at low PLGA content, probably due to high distribution efficiency of internal phase into external phase. Increase in the viscosity of internal phase with increased amount of PLGA also provides resistance for mass transfer during diffusion of internal phase into the external phase leading to particle enlargement.

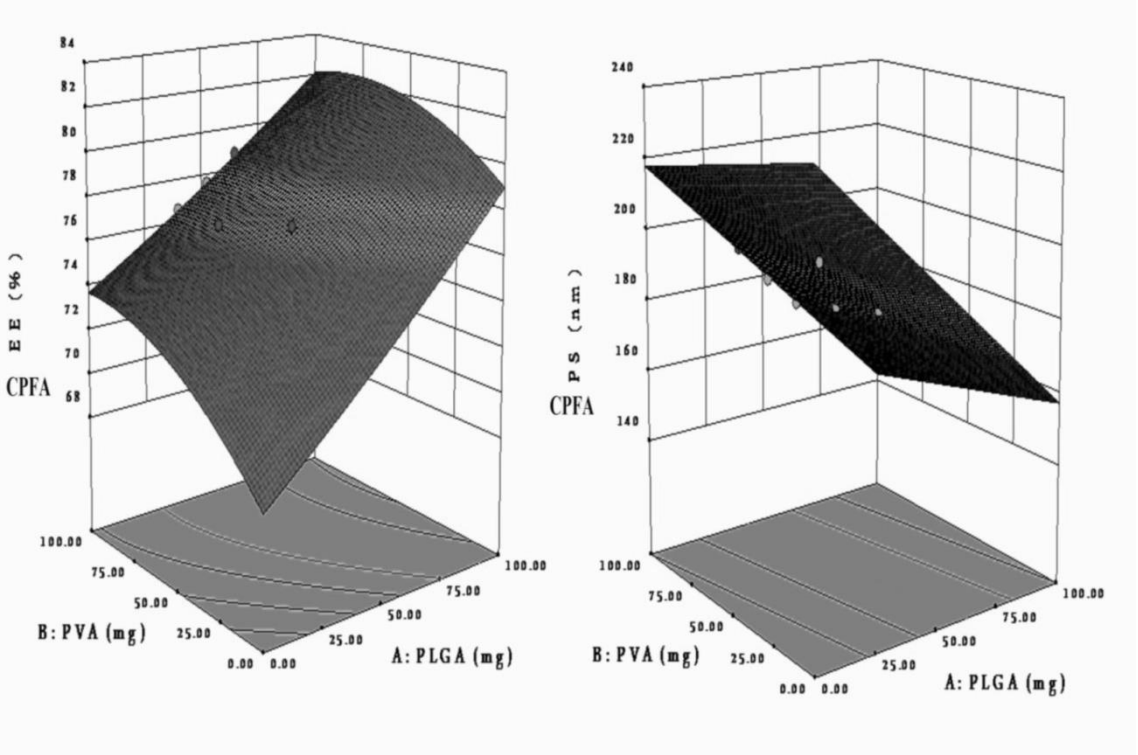
Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.700 was obtained with F5 for CNP having acceptable PS and EE, so F5 for CNP was selected as an optimized formulation and further evaluated for various parameters.

Table 2. The results of multiple regression analysis of the obtained data

Sr. no	**Final Equation in Terms of Coded Factors**		
	EE	**PS**	
1	+76.92	+187.61	=
2	+4.80	-38.00	* X1
3	+2.14	-7.67	* X2
4	-0.58	-	* X1X2
5	+0.27	-	* $X1^2$
6	-1.39	-	* $X2^2$

Number	**PLGA**	**PVA**	**EE**	**PS**	**Desirability**
CNP	55.580	84.690	78.229	178.051	0.700

Figure 1. Response surface plot showing effects of amount of PLGA and PVA (independent variables) on dependent variables PS and EE for CPFA.



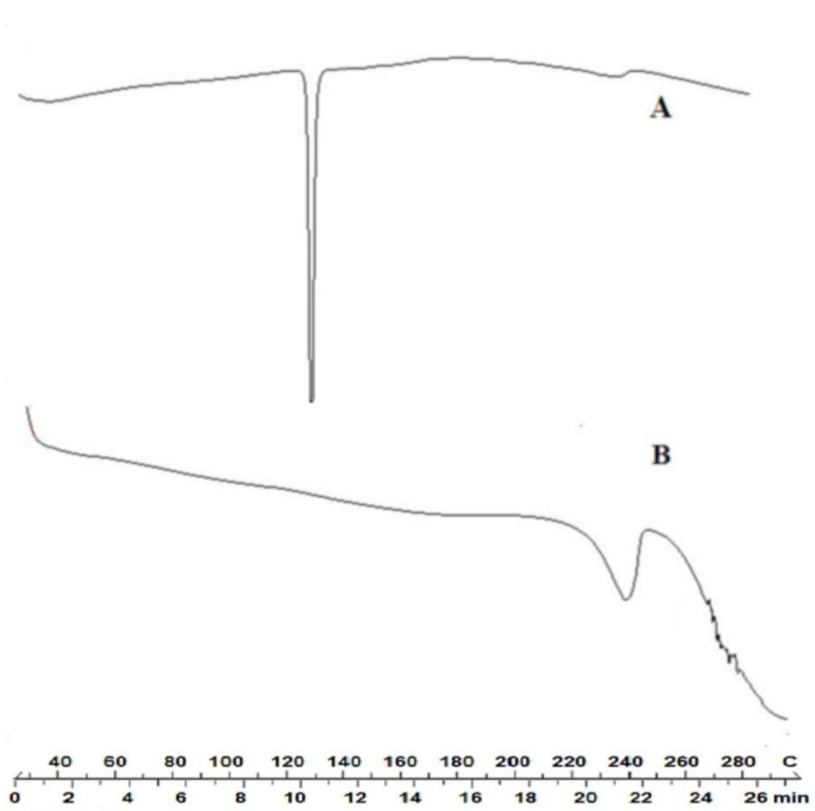
Zeta potential

Zeta potential is essential parameter that gives information about surface charges which has direct influence on colloidal stability and interaction with physiological body cells. The CNP showed negative zeta potential (–15.71 ± 0.65 mV) which may be due to presence of polyvinyl alcohol on surface. The CPFA showed slight shifting of zeta potential towards positive (-12.20 ± 0.44 mV) which may be due to surface modification and amide bond of folic acid. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability and blood clearance.

Differential scanning calorimetry

DSC study was performed for the free CAPE and CPFA (Figure 2) in order to determine the molecular state of the CAPE. A sharp melting transition of free CAPE was observed at 127.48 °C with \H 38.01 J/g which shows transit crystallinity. In CPFA thermogram, the free CAPE peak was disappeared indicating molecular dispersion of CAPE inside polymeric nanoparticles.

Figure 2. Comparative DSC thermo grams of CAPE (A) and CPFA (C)

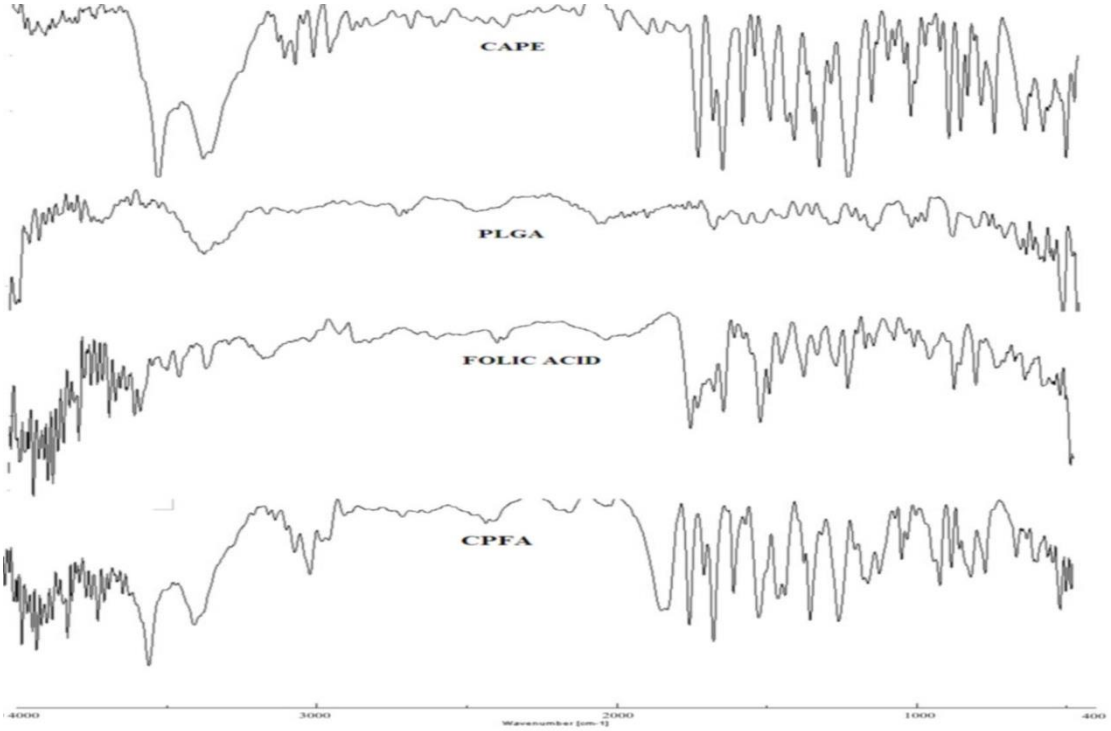


Fourier transform-infrared spectroscopy (FTIR)

Figure 3 shows FTIR spectra of pure CAPE (A), PLGA (B), Folic acid (C) and CPFA (D). It can be seen from the FT-IR spectrum of free CAPE that the bands obtained at 3482 cm^{-1} and 3333 cm^{-1} were assigned to –OH stretching. Band obtained at 3061.44 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1683 cm^{-1}, 1602 cm^{-1}, and 1184 cm^{-1} were also attributed C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PLGA and nanoparticles the bands at 2995 cm^{-1} and 2949 cm^{-1} were C–H stretch of CH_2 and C–H stretch of –C–H–, respectively. A band at 1772 cm^{-1} and 1751 cm^{-1} was assigned to the stretching vibration of C=O of ester bond (strong and narrow) and 1186– 1087 cm^{-1} was attributed to C–O stretching, which belongs to the characteristic peaks of PLGA molecule.

However, in the FTIR spectra of CPFA, the major peaks of CAPE at 3482 cm^{-1}, 3333cm^{-1}, and 1602cm^{-1} were significantly decreased and the presence of these characteristic peaks is a confirmation of CAPE encapsulation on PLGA nanoparticles successfully. Conjugation of folic acid to PLGA nanoparticles was confirmed by the amide bond peak at 1634.38 cm^{-1} in CPFA which clearly indicates the formation of amide bond following conjugation

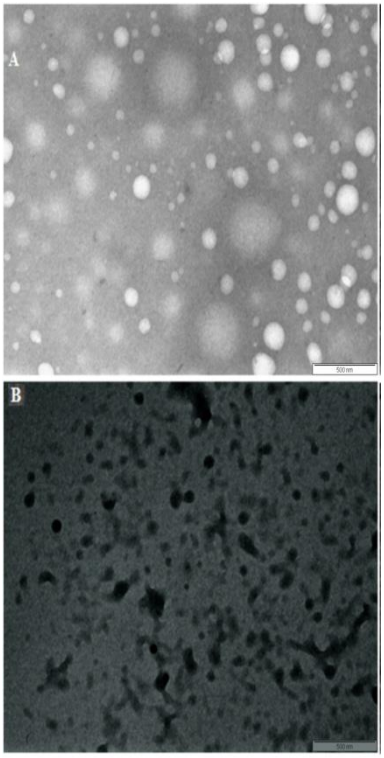
Figure 3. Comparative FTIR spectra of pure CAPE (A), PLGA (B), Folic acid (C), CPFA (D)



Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of the CNP and CPFA was assessed using transmission electron microscope (TEM) from which it can be seen that the nanoparticles were freely dispersed and spherical in shape (Figure 4). In comparison with CNP, CPFA have slight larger particle size. This may be due to surface modification by conjugation of folic acid.

Figure 4. Transmission electron microscopy images of CNP (A), CPFA (B)

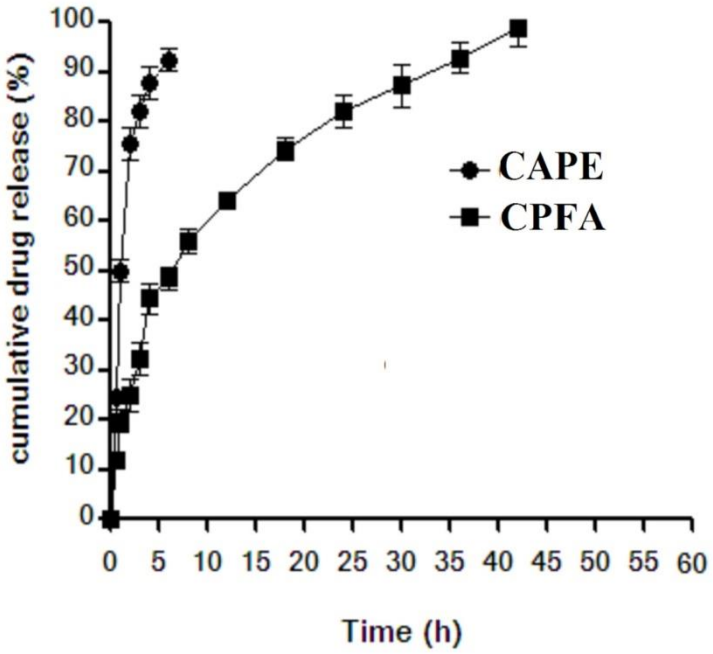


***In vitro* release study**

In vitro release of the CAPE from solution of CPFA was investigated by diffusion bag technique. As CPFA were developed aiming at its intravenous administration, the release studies were conducted phosphate buffer saline (pH 7.4) at 37°C. Figure 5 revealed that CAPE could freely diffuse in its solution form causing 92.39% drug release within 6h. However, CAPE release from polymeric nanoparticles showed a biphasic pattern with initial burst release (19.78%) within the first 1 h followed by sustained release up to 42 h for CPFA.

Erosion, diffusion and degradation are three basic mechanisms contributed for the release of a loaded drug from polymeric nanoparticles. For developed system any or all three mechanisms may be involved in drug release. During the *in vitro* release study, the initial burst release may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a controlled release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Figure 5 the release profile of CAPE from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by a initial rapid release followed by a slower continuous release phase over 54 h.

Figure 5. *in vitro* release of pure CAPE and CAPE from CPFA (A).



***In vitro* cytotoxicity study**

The *in vitro* cytotoxicity study of CPFA was investigated and compared with CAPE against human breast cancer MCF-7 cells using *in vitro* SRB assay.

The results illustrated in Table 3, Figure 6 indicated that CPFA displayed better anticancer activity than CAPE. The total growth inhibition concentration of CPFA (24.80 µg/mL) was approximately 46.09% (46.00 µg/mL) less than the CAPE in solution respectively.

The enhanced anticancer efficacy may be attributed to greater cellular uptake of CPFA via phagocytosis or the fusion process which resulted in the enhanced permeability of the cell membrane to CAPE that allows sufficient drug concentration inside the cells. Therefore, CPFA might have served as a potential nanocarrier to improve *in vitro* anticancer activity of CAPE. The lower anticancer activity of free CAPE in solution may be due to its efflux by P-glycoprotein pumps. The GI50 values for CAPE and CPFA were observed less than 10 µg/mL indicates potent anticancer nature.

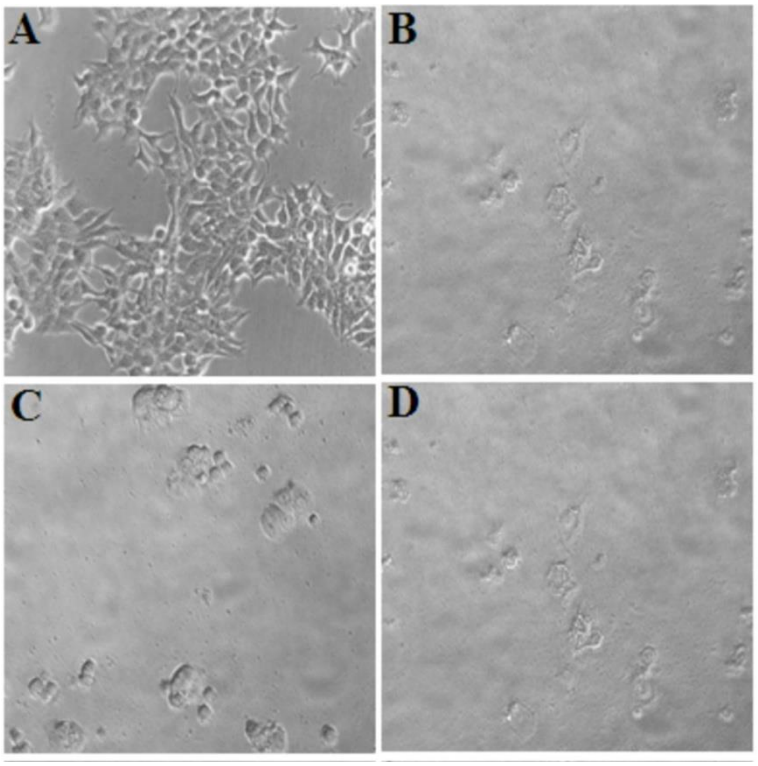
Table 3. TGI and GI_{50} values of CAPE, EEIP, CPFA, EPFA and ADR on MCF-7 breast cancer cell line.

Samples	MCF-7 cell line TGI (µg/mL)	MCF-7 cell line GI_{50} (µg/mL)
CAPE	46.00	12.1
CPFA	24.8	< 10
ADR	< 10	< 10

Values were presented as mean ± SD (n = 3).

TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produce 50% inhibition of cells, MCF-7 breast cancer cell line

Figure 6. *In vitro* cytotoxicity study on MCF-7 Cell lines (A) MCF-7 control (B) Positive control ADR (C) CAPE (D) CPFA Treated.



Hemolysis study

For *in vivo* application it is necessary to evaluate the biosafety by hemolysis study. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate for up to 1.5 mg/mL concentration of CAPE and CPFA were 2.5 and 2.2, % respectively. The acceptable hemolysis rate (less than 3%) [23] Shown denotes formulations having non hemolytic property up to 1.5 mg/mL and may be considered as biosafe for internal use.

***In vivo* anticancer study**

Effect on Tumor Growth, Hematological Parameters and Biochemical Parameters

In the DLA tumor control group, the average life span of animal was found to be 48%. Treatment with CAPE at a dose of 50,100,200 mg/kg increase in body weight and life span was observed as shown in table 4. whereas CPFA nanoparticle formulation at a dose of 100,200,400 mg/kg body weight increase the life span to 91%, 92%, 93%, respectively. In case of CPFA more percentage increase in life span was observed that may be because of increased solubility of CAPE when formulated as nanoparticles system. These values were significantly different from normal control (G_1) at $P < 0.01$ and cancer control (G_2) at $P < 0.01$. However the average life span of 5-FU treatment was found to be 96%, indicating its potent antitumor nature. The antitumor nature of CPFA nanoparticle formulation at a dose of

100,200,400 mg/kg was evidenced by the significant reduction in percent increase in body weight of animal when compared to DLA tumor bearing mice. It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in CPFA nanoparticle formulation at a dose of 100,200,400 mg/kg treatments when compared to the DLA tumor control. (Table 4 & 5). In case of CPFA more reduction in packed cell volume and viable Tumor cell count was observed that may be due to increased solubility of CAPE. Due to nanoparticles formulation, drug availability might have increased on site of action because of achieving EPR

Table 4. Effect of CPFA on the life span, body weight and cancer cell count of tumor induced mice.

Treatment Groups	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G_1	6	>>30 days	2.22±0.68	-
G_2	6	48%	$9.44±1.86^{a**}$	$2.75±0.80^{a**}$
G_3	6	96%	$5.66±0.42^{b**}$	$1.30±0.22^{b**}$
G_4	6	82%	$6.22±0.90^{b**}$	$1.65±0.45^{b**}$
G_5	6	84%	$5.80±0.80^{b**}$	$1.50±0.40^{b**}$
G_6	6	86%	$5.60±0.40^{b**}$	$1.46±0.36^{b**}$
G_7	6	91%	$5.26±0.26^{b**}$	$1.35±0.20^{b**}$
G_8	6	92%	$5.05±0.20^{b**}$	$1.34±0.18^{b**}$
G_9	6	93%	$4.96±0.16^{b**}$	$1.33±0.15^{b**}$

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 toG6– Treatment control (CAPE 50,100,200 mg/kg) G7to G9 – Treatment control (CPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

As shown in (Table 5) RBC, Hb, platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with CPFA nanoparticle formulation at a dose of 100,200,400 mg/kg significantly increases the Hb content, RBC, platelets and significantly decreased the WBC count to about normal level. From the results it can be concluded that the CPFA nanoparticle formulation showed comparatively better effects on hematological parameters that showed the developed system is biocompatible with less hemolysis.

Table 5. Effect of CPFA on Hematological parameters

Treatment Groups	Total WBC Cells /mlx10^3	RBC Count millions/mm^3	Hb gm/dl	PCV %	Platelets Lakhs/ mm^3
G_1	10.35 ±1.05	4.55±1.95	12.90 ±1.95	14.25±2.44	3.60±0.95
G_2	15.30 ±2.60^{a**}	2.70±0.98^{a**}	6.80 ±0.95^{a**}	38.36±3.35^{a**}	1.70±0.42^{a**}
G_3	12.30 ±1.34^{b**}	4.05±1.62^{b**}	11.90±1.48^{b**}	16.40±1.40^{b**}	2.94±0.50^{b**}
G_4	13.24 ±1.85^{b**}	3.75±1.10^{b**}	12.05±1.40^{b**}	18.65±2.90^{b**}	3.10 ±0.54^{b**}
G_5	12.95±1.64^{b**}	3.90±1.22^{b**}	12.10±1.44^{b**}	17.45±2.45^{b**}	3.18±0.58^{b**}
G_6	12.40±1.44^{b**}	4.03±1.46^{b**}	12.16±1.48^{b**}	17.38±2.40^{b**}	3.25±0.62^{b**}
G_7	11.74±1.08^{b**}	4.22±1.72^{b**}	12.42±1.64^{b**}	17.08±2.20^{b**}	3.44±0.76^{b**}
G_8	11.60±1.05^{b**}	4.28±1.78^{b**}	12.46±1.66^{b**}	16.75±2.05^{b**}	3.48±0.78^{b**}
G_9	11.52±1.02^{b**}	4.32±1.86^{b**}	12.52±1.70^{b**}	16.70±1.92^{b**}	3.55±0.82^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 toG6– Treatment control (CAPE 50,100,200 mg/kg) G7to G9 – Treatment control (CPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

The inoculation of DLA cells caused significant increase in the level of total Cholesterol, Aspartate amino Transferase, Alanine amino Transferase and Alkaline Phosphatase in the tumor control animals(G2), when compared to the normal group. The treatment with CPFA nanoparticle formulation at a dose of 100,200,400 mg/kg body weight reversed these changes towards the normal level (Table 6).

Table 6. Effect of CPFA on serum Enzymes and lipid proteins

Treatment Groups	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)

G_1	108.85±3.05	136.85±2.55	36.40 ±1.65	31.28 ±1.45	132.28 ±2.08
G_2	146.95±4.34[a**]	220.28±4.40[a**]	78.6±2.94[a**]	62.32±2.60[a**]	265.30±4.35[a**]
G_3	126.30±3.84[b**]	169.15±2.65[b**]	44.40 ±1.72[b**]	34.52±1.70[b**]	154.45±2.40[b**]
G_4	124.28±3.65[b**]	173.40±2.95[b**]	50.45±2.40[b**]	37.40 ±1.75[b**]	173.40±2.46[b**]
G_5	122.24±3.55[b**]	169.65±2.73[b**]	46.52 ±2.38[b**]	36.30±1.65[b**]	165.30±2.36[b**]
G_6	119.30±3.50[b**]	165.60±2.62[b**]	44.50 ±2.32[b**]	35.34±1.60[b**]	163.12±2.30[b**]
G_7	112.40±3.24[b**]	150.32±2.40[b**]	40.85 ±2.12[b**]	34.35±1.25[b**]	155.80±2.10[b**]
G_8	111.30±3.16[b**]	148.15±2.36[b**]	40.65 ±2.05[b**]	33.84±1.20[b**]	154.35±2.05[b**]
G_9	110.20±3.08[b**]	145.80±2.30[b**]	39.50 ±2.02[b**]	33.60±1.05[b**]	152.34±2.00[b**]

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 toG6– Treatment control (CAPE 50,100,200 mg/kg) G7to G9 – Treatment control (CPFA 100,200,400 mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from normal control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Stability studies

CPFA were subjected to stability study at 40 ± 2°C/75 ± 5% RH. No significant difference in EE and PS were observed over the period of three months as compared to initial values of freshly prepared nanoparticle formulations. *($p > 0.05$)*

Table 7. Accelerated stability study of CPFA for 3 months

Time (Months)	PS (nm)	Percentage of EE
	CPFA	CPFA
Initial	189±3	76.62±1.18
1	191±2	75.25±0.94
2	192±2	74.02±1.08
3	194±3	73.62±0.94

Values were presented as mean ± SD (n = 3).

Data was analyzed by one way ANOVA followed by Dunnett's multiple comparison test with $p < 0.05$.

Conclusion

We have investigated folic acid conjugated nanoparticulate formulation composed of PLGA and PVA as an injectable nano carrier for the delivery of a lipophilic anticancer bioactive, CAPE. The developed formulation displayed smaller particle size, excellent encapsulation efficiency and control release with better stability. CPFA demonstrated increase in anticancer efficacy as compared to CAPE. CPFA demonstrated higher *in vitro* as well as *in vivo* anticancer activity in human breast cancer MCF-7 cells than its free form, which may leads to reduction in dose as well as cost. Developed formulation can be considered as an alternative dosage form for CAPE to improve the biopharmaceutical properties and to enhance therapeutic efficacy in cancer chemotherapy.

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Research Article

Design and Development of Indian Propolis Loaded Poly (ε -Caprolactone) Nanoparticles For Improved Anticancer Efficacy

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ABSTRACT

Propolis, a natural product obtained from apiculture and is well proven for its anticancer potential. Anticancer efficacy of propolis is limited to its poor water solubility and bioavailability. The present study was designed and investigated for development of ethanolic extract of Indian propolis (EEIP) loaded Poly (ε-caprolactone) nanoparticles (EPL) to achieve improved solubility, sustained drug release and enhanced cytotoxic efficacy of EEIP. Formulation development, characterization and optimization were carried out by design of experiment approach. Developed formulations were evaluated in detail for nanoparticle characterization and *in vitro* cytotoxicity study. Developed nanoparticles showed particle size, and encapsulation efficiency of 190 ± 1 - 230 ± 2 nm and 62.66± 1.20 - 67.18± 1.80 % respectively. Optimized formulation EPL showed sustained drug release over a period of 48 h. Concentration of the drug needed for total growth inhibition of cells in a designed time period (TGI) was decreased by 33.06% for EPL as compared to EEIP in human breast cancer MCF-7 cells and 22.31% in human colon cancer cells HT-29 indicating improved cytotoxicity of EEIP. The study proven that the optimized EPL exhibited increased solubility, sustained drug release and enhanced *in vitro* cytotoxicity on MCF-7 and HT-29 cell lines in comparison with EEIP. Thus developed system may be served as potential nanocarrier in anticancer treatment.

Keywords : Indian propolis; Poly (ε-caprolactone); Design of experiment; Sulforhodamine B (SRB) assay

INTRODUCTION

Propolis, a natural honey bee product contains various polyphenols and flavonoids in which Caffeic acid phenethyl ester is most active polyphenolic constituent responsible for anticancer activity [1]. Propolis of various regions is known to exhibit various activities including antifungal, antibacterial and anticancer activity [2-4]. Various in vitro and in vivo anticancer studies on propolis and its isolated constituents have been reported for anticancer activity [5-7]. Although propolis has beneficial anticancer activity on human cancer cell lines but it has some limitations such as it is high lipophilicity, poor solubility in water. The overall effect leads to low bioavailability and poor biological activity. The solubility is an important factor on which drug dissolution, absorption and bioavailability are directly depends. The biological effects of drugs get affected because of its low solubility. Various approaches are reported in order to increase drug solubility including cyclodextrin complex formation solid dispersions or some chemical modifications. But there are various limitations associated with these approaches [8]. Polymeric nanoparticle system is one of the approaches established to overcome the limitations. The biodegradable polymeric nanoparticles have been investigated especially for achieving increased drug solubility, drug targeting to cancer cells due to their small particle size (10–1000 nm) by enhanced permeability and retention effect, prolonging the biological activity by extending blood circulating time and increasing drug concentration and residence time of drug at specific site of body [9]. Poly (ε-caprolactone) (PCL) is one of the biodegradable polymer most commonly used for pharmaceutical applications [10-12]. PCL has various advantages such as biocompatibility, biodegradation and applicability in formulation development [13]. PCL has been used extensively for developing nanoparticulate drug delivery systems because of its various advantageous properties such as high hydrophobicity, high permeability, and biodegradability. Its biodegradation end product 6-hydroxycaproic acid is neutral, which does not disturb the pH balance [14]. Quality by design (QbD) approach helps researchers to understand the effects of critical variables in formulation and process on the product quality. Design of Experiment (DoE) is a key factor involved in QbD. Nanoparticle formulations can be developed using DoE approach to identify critical process parameters, reduce number of experiments, and study the interactions between formulation variables [8]. Some attempts have been reported for development of nanoparticulate formulations of Indian propolis for antimicrobial and anticancer drug delivery but it lacks detailed investigation of formulation parameters and

characterization [15, 16, 17, 18]. In addition, natural products are known for their activities through synergistic mechanisms so prompted by the above facts, the present study has been designed to develop standardized ethanolic extract of Indian propolis (EEIP) loaded PCL nanoparticles (EPL) using Design of Experiment approach to investigate its formulation characteristics and in vitro anticancer efficacy.

Materials and methods

Materials

Pluronic® F-68, PCL (poly-ε-caprolactone, mw 65,000 Da), and dialysis bag with a 12,000 molecular weight cut off, were purchased from Sigma-Aldrich Chemical Private Ltd (Bangalore, India). Acetone GR grade, sodium hydroxide and potassium dihydrogen phosphate were purchased from Merck India. All other chemical reagents used were of pharmaceutical grade.

Extraction and characterization of Indian propolis

EEIP was prepared by extraction of crude propolis (10g) pre-treated with hexane and ethyl acetate by sonication method. Further extraction was done with ethanol by soxhlet method at 60^0C to obtain ethanolic extract of Indian propolis (EEIP). The obtained EEIP was standardized for CAPE using developed high performance liquid chromatography (HPLC) method and stored at 2-8^0 C.

Preparation of EEIP loaded polymeric nanoparticles

EEIP was dissolved in 3 ml of acetone containing PCL polymer and Pluronic® F-68 as per composition showed in Table 1. Organic solution was injected into 10 ml distilled water at a rate of 10 ml/min under magnetic stirring at 2000 rpm for 1 h at 40°C. Resultant aqueous nanoparticle suspension was filtered through 0.45 μ filter to remove copolymer aggregates. Concentrations of PCL and Pluronic® F-68 (PF 68) were also optimized and selected to obtain stable suspension [9].

Table 1 The effect of various formulations (with different amounts of PCL and PF68) on PS and EE by 3^2 factorial design with coded levels and actual amount values of variables.

Batches EPL	Coded levels (X_1, X_2)	Amt. of PCL (mg; X_1)	Amt of PF 68 (mg; X_2)	Drug Content (%)	Nanoparticle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)
F1	+1, +1	62.50	100.00	94.37± 0.90	230± 2	65.71± 1.20
F2	+1, 0	62.50	75.00	94.73± 0.45	226± 2	64.33± 1.20
F3	+1,-1	62.50	50.00	95.83± 0.45	220± 2	64.23± 1.80
F4	0, +1	50.00	100.00	93.42± 0.90	203± 1	65.42± 1.20
F5	0, 0	50.00	75.00	93.61± 0.75	200± 2	66.61± 1.80
F6	0, -1	50.00	50.00	93.80± 0.75	199± 2	67.18± 1.80
F7	-1, +1	37.50	100.00	92.62± 0.45	198± 3	63.62± 1.80
F8	-1, 0	37.50	75.00	92.62± 0.90	195± 2	63.20± 1.45
F9	-1, -1	37.50	50.00	92.36± 0.45	190± 1	62.66± 1.20

Values were presented as mean ± SD (n = 3).

Particle size analysis, Drug content and Encapsulation Efficiency (EE)

The particle size of developed formulations was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average particle size was determined, expressed in terms of d (0.9) μm.The concentration of active ingredients (drug content and encapsulation efficiency) in the polymeric nanoparticle formulation was measured by high performance liquid chromatography method (HPLC, Jasco UV 2057, Japan). A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 ml/min using BDS Hypersil C_{18} (250 mm x 4.6 mm; 5 μ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 μl, and the detection wavelength was set at 331 nm. Temperature was maintained at 25^0± 2^0 C. The nanoparticle solution was suitably diluted with alcohol prior to determination. The percent drug content was calculated according to the equation:

Drug Content (%) = (Amount of EEIP in nanoparticle/ Amount of EEIP and polymer) × 100 (1)

Encapsulation efficiency was determined by separating non-encapsulated active ingredients from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4^0C. The sediment nanoparticles were disrupted with acetone to release the entrapped EEIP; suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method, (Jasco UV 2057, Japan) The percent EE was calculated using Equation (2).

Encapsulation efficiency (%) = Amount of EEIP entrapped in the nanoparticle/ Initial amount of the EEIP added × 100. (2)

Design of experiments DoE approach

For determination of optimized nanoparticle formulation Design of experiment approach was used. Particle size and encapsulation efficiency values were measured after collecting the nanoparticles. For the statistical design, Design Expert® Version 10.0 was used and particle size and encapsulation efficiency were selected as dependent

variables while the amount of polymer (PCL) and amount of surfactant (PF 68) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of system were performed.

Optimization by 3^2 factorial design

Preliminary experiments helped in understanding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PCL (X1) and PF 68 (X2) concentration were identified as crucial factors in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was adopted to optimize the nanoparticles composition while studying the effect of X1 and X2 (independent variables) on Particle size and encapsulation efficiency (two dependent variables or responses) by obtaining a 3D response surface plot.

Zeta potential

The zeta potential study was carried out with the laser Doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments) at a temperature of 25°C.

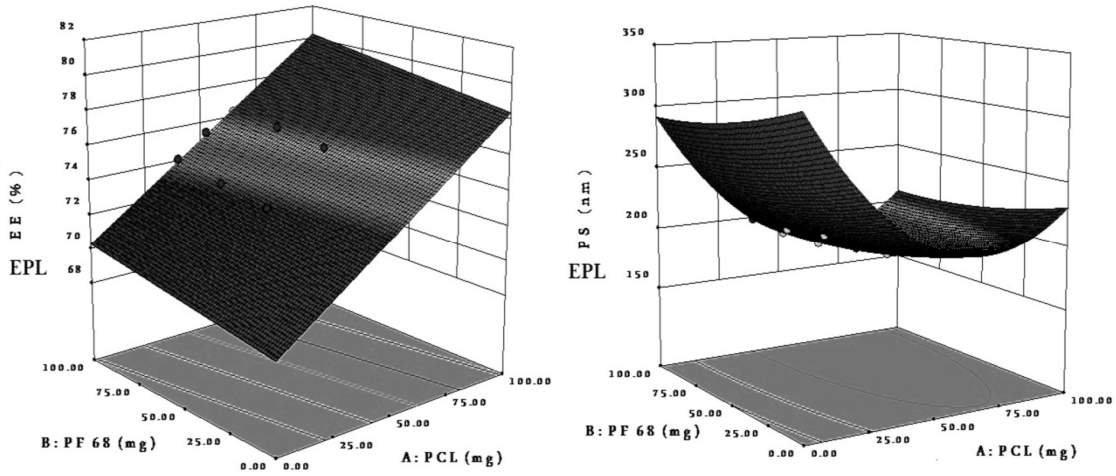

Fig.1. Response surface plot showing effects of amount of PCL and PF 68 (independent variables) on dependent variables Particle size and encapsulation efficiency for EPL

Differential scanning calorimetry

The differential scanning calorimetry (DSC) thermograms of EEIP, PCL and EPL (vacuum-evaporated samples) were obtained using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples were (5 mg) heated in hermetically sealed aluminium pan with a heating rate of 10°C/min under a nitrogen atmosphere (flow rate 50 ml/min).

Fourier transform-infrared spectroscopy (FTIR)

FTIR spectra of EEIP, PCL and EPL were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan) upon mixing in dry KBr. Each sample was scanned in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of nanoparticle suspensions was studied using transmission electron microscopy (TEM). A drop of EPL was applied to a carbon film-covered copper grid. Most of the suspension was blotted from the grid with filter paper to form a thin film specimen. The unstained sample was then examined and photographed with a Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV and a magnification of 50000X.

In vitro drug release study

Polymeric nanoparticle formulation (5 ml) was placed into a dialysis bag (cut off 12,000D).The bag was immersed into a beaker containing phosphate buffer (pH 7.4) at 37 ± 0.5 °C with continuous magnetic stirring at 100 rpm. At predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36 and 42h) the sample (5 ml) was withdrawn maintaining sink condition. The samples were analyzed for EEIP content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of EEIP was plotted against time. Pure EEIP release was analyzed in a similar manner [19].

In vitro cytotoxicity studies

Cytotoxic activity of EEIP and EPL was evaluated on human breast cancer MCF-7 and human colon cancer HT-9 cell line using sulforhodamine B (SRB) assay. The study performed as per the procedure described by Bothiraja et al, 2013 at Tata Memorial Center, Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Formulations were diluted in distilled water so as to obtain EEIP in the concentration ranges of 10, 20, 40 and 80μg/ml which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^3 cells per well in 96-well plates using in-situ fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37ºC with 100% relative humidity (RH), the growth medium was replaced with 100 μl of fresh medium containing various concentrations (10-80 μg/ml) of EEIP and EPL. The culture media without any drug formulation

was used as a control. After 48 h incubation, assay was terminated by adding 50 µl of the cold TCA and incubated for 60 min at 4ºC. The media was removed and washed with sterile PBS and dried. 50 µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation.

Cell growth (%) = (Average absorbance of the test well / Average absorbance of the control wells) ×100 [20].

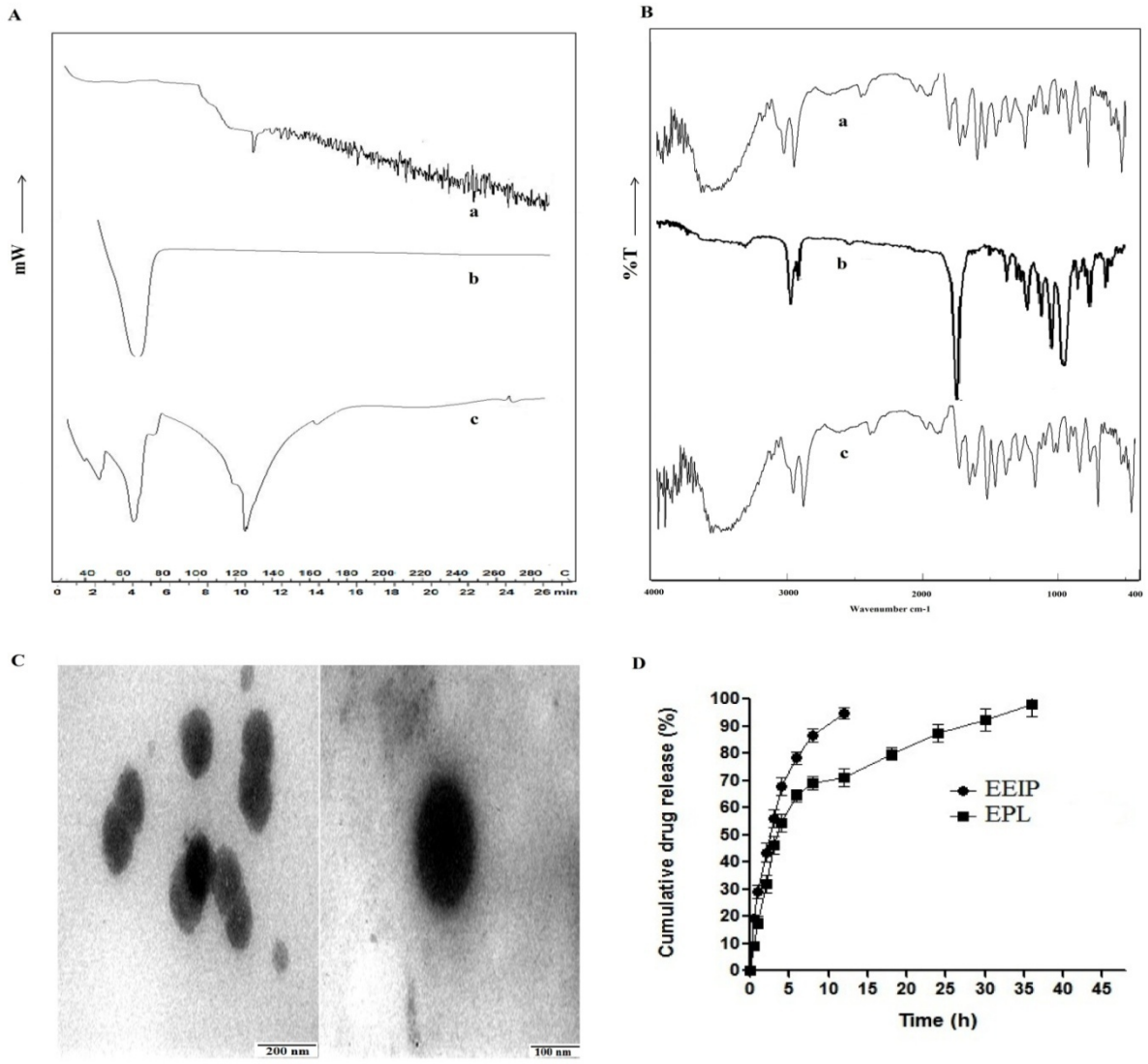

Fig. 2 A. Comparative DSC thermo grams of EEIP (a), PCL (b), EPL (c) B. Comparative FTIR spectra of EEIP (a), PCL (b), and EPL (c), C. Transmission electron microscopy images of EPL D. *in vitro* release of pure EEIP and EEIP from EPL

Hemolysis study

The hemolytic effects of the EEIP and EPL were determined using the method described by love et al. 2012. Briefly, fresh 10 ml blood collected from healthy donors and fibrinogen was removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. after removal of supernatant the precipitate diluted with aqueous NaCl solution (1:50 v/v) to get 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number represented a set of three tubes. To all of the tubes 2.5 ml of the red blood cell suspension was added. The tubes labeled 1 were diluted with 2.5 ml of distilled water as the hemolysis control (100% hemolysis) and the tubes labeled 2 were diluted with 2.5 ml of 0.9% isotonic NaCl solution as the non hemolysis control (0% hemolysis). The remaining tubes were diluted with

EEIP and EPL samples corresponding to concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/ml of formulations and volume made up to 5 ml with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer [21]

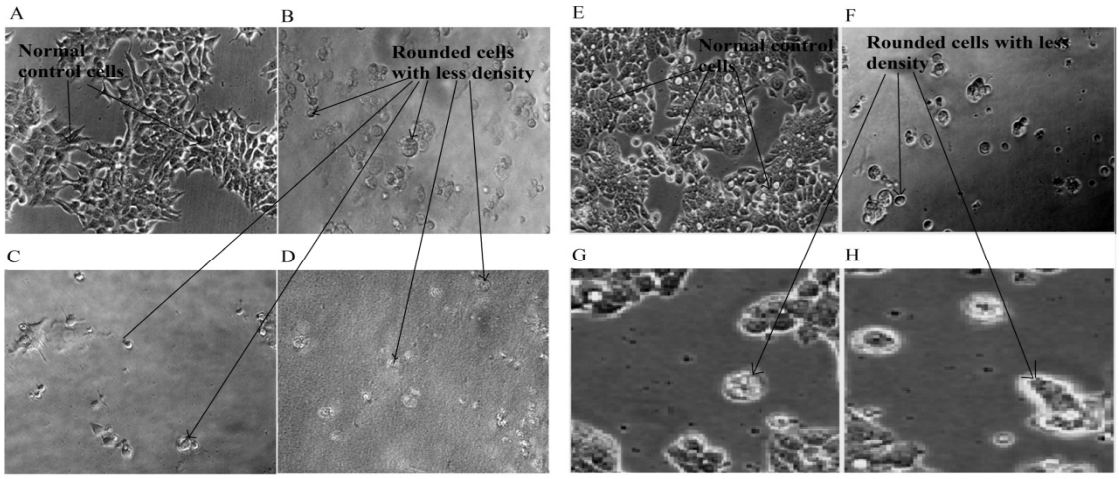

Fig. 3 *In vitro* cytotoxicity study on MCF-7 Cell lines (A) Normal control (MCF-7) (B) Positive control ADR (C) EEIP Treated (D) EPL Treated and *In vitro* cytotoxicity study on HT-29 Cell lines (E) Normal control (HT-29) (F) Positive control ADR (G) EEIP Treated (H) EPL Treated

Stability study

EPL was transferred in glass vial and subjected to stability study at 4 °C in refrigerated condition. Optimized nanoparticles were analyzed for the change in Particle size and encapsulation efficiency upto 180 days.

Statistical analysis

The results were expressed as mean ± S.D. For the statistical design, Design Expert® Version 10.0 was used.

Results and discussion

In present study attempt has been made to develop EEIP loaded nanoparticulate formulations by considering its safety and anticancer efficacy on breast cancer and colon cancer cell lines. Nanotechnolgy based drug delivery systems has proven for improving the solubility, efficacy and safety of various drugs over the past few decades [22, 23]. In this study, EEIP loaded polymeric nanoparticles have been developed and investigated as an nanocarrier in order to improve its solubility, to achieve sustained release and improve in vitro anticancer efficacy. The effect of the EEIP loaded polymeric nanoparticles composition on Particle size and encapsulation efficiency was studied by design of experiments (DoE) approach.

Particle size, drug content and encapsulation efficiency

The mean EPL nanoparticles size was in the range of 190 ± 1 - 230 ± 2 nm and was strongly affected by the selected variables. The drug content and encapsulation efficiency were in the range of 92.36± 0.45 to 95.83± 0.45% and 62.66± 1.20 - 67.18± 1.80 % respectively for EPL (Table 1). A good fit (r^2 for PS = 0.9941 and EE = 0.8292 for ENP) was observed for the particle size and encapsulation efficiency the independent variables. Drug delivery to specific sites of the body is influenced by size of the nanoparticles; smaller particles may tend to minimize the particle uptake by non targeted cells, including their premature clearance by the mononuclear phagocytic system [24].

Optimization of EPL nanoparticles by 3^2 factorial design

Particle size and encapsulation efficiency are the major important factors considered in formulation development of nanoparticles. During the preliminary study, assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of polymer and surfactant (Table 1). EEIP content was kept constant. As per 3^2 factorial design, nine different batches were prepared. The responses of these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using Design Expert® Version 10.0. The results of multiple regression analysis of the obtained data are summarized in Table 2. From the factorial design study of EPL (Table 2), we observed that positive coefficients of the main terms X1 and X2 indicated a favorable effect on the mean particle size and encapsulation efficiency with the PCL and PF68. The PCL had a linear effect on the particle size and curvilinear effect on encapsulation efficiency while PF 68 had linear effects on both Particle size and encapsulation efficiency as seen in surface plot (Fig.

1). Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.700 was obtained with F3 so it was selected as an optimized formulation and further evaluated for various parameters.

Table 2 : Results of multiple regression analysis of the obtained data

Sr. no	Final Equation in Terms of Coded Factors		
	EE	PS	
1	+63.21	+191.06	
2	+22.23	+13.33	* X1
3	+0.39	+7.67	* X2
4	+1.04	+4.00	* X1X2
5	-39.12	+146.67	* $X1^2$
6	+0.36	-1.33	* $X2^2$

Zeta potential

Zeta potential is essential parameter gives information about surface charges surface charges which has direct influence on colloidal stability and interaction with physiological body cells. The EPL showed negative zeta potential (–26.44 ± 0.10 mV) which may be due to presence of PF 68 on surface. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability and blood clearance.

Differential scanning calorimetry

Study was performed for the free EEIP, PCL and EPL (Fig. 2A) in order to determine the molecular state of the EEIP. A sharp melting transition of free EEIP was observed at 127.48 °C with \H 38.01 J/g shows transit crystallinity. In EPL thermogram, the free EEIP peaks were disappeared indicating molecular dispersion of EEIP inside polymeric nanoparticles.

Fourier transform-infrared spectroscopy (FTIR)

Fig. 2B shows FTIR spectra of pure EEIP (a), PCL (b) and EPL (c). It can be seen from the FT-IR spectrum of free EEIP showed various peaks at 3571.52 cm−1, 3541.63 cm−1, 3485.7 cm−1 and 3333 cm−1 that were assigned to –OH stretching. Band obtained at 3083.62 cm -1 was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm−1, 1594.8 cm−1, and 1164.7 cm−1 were attributed to C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PCL the bands at 2940 cm^{-1} and 2860 cm^{-1} were C–H hydroxyl groups asymmetric stretching and C-H hydroxyl groups symmetric stretching respectively. 1722 cm^{-1} is assigned to C=O stretching vibrations of the ester carbonyl group. The absorption at 1238 cm^{-1} is assigned to C-O-C asymmetric stretching and 1160 cm^{-1} is C-O-C symmetric stretching. However, in the FTIR spectra of EPL the major peaks at 3571.52 cm−1, 3541.63 cm−1 were assigned to –OH stretching. Band obtained at 3083.62 cm -1 was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm−1, 1594.8 cm−1, and 1164.7 cm−1 were significantly decreased by its intensity and the presence of these characteristic peaks is a confirmation of EEIP encapsulation on PCL nanoparticles successfully.

Transmission electron microscopy (TEM) of nanoparticles

Surface morphology of the EPL was assessed using transmission electron microscope (TEM) from which it can be seen that the nanoparticles were freely dispersed and spherical in shape (Fig. 2C). Little bigger size nanoparticles were observed in TEM than that tested by laser diffraction technique. Because of low melting points of PF 68 (~55 °C) and PCL (~60 °C), nanoparticles may undergoes the melting due to high energy electron beam in TEM. Melting might cause expansion to certain level and nanoparticles seem bigger in TEM.

In vitro release study

In vitro release of the EEIP from solution of EPL was investigated by diffusion bag technique. Release studies were conducted in phosphate buffer saline (pH 7.4) at 37°C. Fig. 2D revealed that EEIP could freely diffuse in its solution form causing 96.79% drug release within 6 h respectively. However, EEIP release from polymeric nanoparticles showed a biphasic pattern with initial burst release (18.00%) within the first 1 h followed by sustained release up to 36 h for EPL. Erosion, diffusion and degradation are three basic mechanisms contributed for the release of a loaded drug from polymeric nanoparticles. For developed system any or all three mechanisms may be involved in drug release. During the in vitro release study, the initial burst release may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a controlled release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Fig. 2D the release profile of EEIP from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by a initial rapid release followed by a slower continuous release phase over 36 h.

In vitro anticancer activity

The in vitro anticancer activity of EPL was investigated and compared with EEIP against human breast cancer MCF-7 cells and human colon cancer cells HT-29 using in vitro SRB assay. The results obtained from study indicated that EPL displayed superior anticancer activity than EEIP on breast cancer MCF-7 cells. The total growth inhibition concentration of EPL was observed 20.5 $\pm$ 0.001 μg/ml while for EEIP it was 30.1 $\pm$ 0.015 μg/ml. In case of colon cancer HT-29 cells lines EPL displayed superior anticancer activity than EEIP. The total growth inhibition concentration of EPL was observed 31.00 $\pm$ 0.020 μg/ml and for EEIP 39.9 $\pm$ 0.020 μg/ml.

Results and microscopic images showed that in Fig. 3 A & E were appeared more dense which are of normal control group of MCF-7 and HT-9 respectively. In Fig. 3 B & F is of positive control group treated with Adriamycin, Fig.3 C & G were images of EEIP and EPL treated group on MCF-7 cells respectively and appearing to be less dense and rounded. Similarly Fig.3 D & H were images of EEIP and EPL treated group on HT-29 cells respectively and appearing to be less dense and rounded The enhanced anticancer efficacy may be attributed to greater cellular uptake of EPL via phagocytosis or the fusion process which resulted in the enhanced permeability of the cell membrane to EEIP that allows sufficient drug concentration inside the cells. Therefore, EPL might serve as a potential nanocarrier to improve in vitro anticancer activity of EEIP. The lower anticancer activity of free EEIP in solution may be due to its efflux by P-glycoprotein pumps. The GI50 values for EEIP and EPL were observed less than 10μg/ml indicates potent anticancer nature on both cell lines.

Hemolysis study

For in vivo application it is necessary to evaluate the biosafety by hemolysis study. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate for up to 1.5 mg/ml concentration of EEIP and EPL were 2.8 and 2.7, % respectively. The acceptable hemolysis rate (less than 3%) [21] denotes formulations having non hemolytic property up to 1.5 mg/ml and may be considered as biosafe for internal use.

Stability studies

No significant difference in EE and PS were observed over the period of 180 days as compared to initial values of freshly pr

Conclusion

We have investigated nanoparticulate formulation composed of PCL and PF-68 as an nano carrier for the delivery of an anticancer bioactive, EEIP. Developed formulation displayed smaller particle size, excellent encapsulation efficiency and sustained release with better stability. EPL demonstrated increase in anticancer efficacy as compared to EEIP. EPL demonstrated higher in vitro anticancer activity in human breast cancer MCF-7 cells and human colon cancer HT-29 than its free form, which may leads to reduction in dose. Developed formulation can be considered as an alternative dosage form for EEIP to improve the biopharmaceutical properties and to enhance therapeutic efficacy in cancer chemotherapy.

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Research Article

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Indian Propolis Loaded Folic Acid Conjugated PLGA Nanoparticles: Formulation Development, Characterization, *In Vitro* and *In Vivo* Anticancer Study

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Abstract

Propolis, a natural bee hive product is well proven for its anticancer potential due its various polyphenols and flavonoids constituents. Anticancer efficacy of propolis is limited due to its poor water solubility and bioavailability. The present study is investigated for design and development of ethanolic extract of Indian propolis (EEIP) loaded folic acid conjugated Poly (D,L-lactide-co-glycolide) nanoparticles (denoted as ELFPN) were investigated to achieve improved solubility, sustained drug release and to study synergized anticancer efficacy. Formulation development, characterization and optimization were carried out by design of experiment approach. . *In vitro* and *in vivo* cytotoxicity study was carried out for optimized formulation. Developed ELFPN showed the particle size and encapsulation efficiency 178 ± 5 - 205 ± 5 nm and 73.16 ± 1.89 - 76.37 ± 1.89 respectively. Optimized formulation showed sustained drug release over a period of 48 h with no sign of blood toxicity. Moreover, concentration of the drug needed for growth inhibition of 50 % of cells in a designed time period (GI50) was decreased by 43.34 %, for ELFPN as compared to EEIP in human breast cancer MCF-7 cells indicating targeting with synergistic effect of ELFPN. An improved anticancer effect was reflected in *in-vivo* Daltons Ascites Lymphoma model by reducing tumor cells count. The developed ELFPN showed improved *in vitro* cytotoxic effect, *in-vivo* anti-cancer activity with desirable characteristics for nanoparticle formulation thus can be useful for biomedical applications.

Keywords

Indian propolis; Formulation; Nanoparticles; PLGA; Conjugation; Factorial design; Cancer

Introduction

Propolis, a resinous material collected by honey bees specifically *Apis mellifera* from the various plant parts, exudates and buds is known for its nutritional and medicinal properties obtained from apiculture [1]. Apart from use of propolis as a nutritional and healthy food constituent, its use in human health in folk medicine [2] has been reported from ancient time. Propolis of various regions is known to exhibit various activities including antifungal, antibacterial and anticancer activity [3-5]. Various *in vitro* and *in vivo* anticancer studies on propolis and its isolated constituents have been reported for anticancer activity [6-8]. Caffeic acid phenethyl ester (CAPE) is an important active chemical constituent of honeybee propolis which is chemically poly phenolic compound [9,10]. Anticancer activity of Indian propolis is limited due to its high lipophilicity and poor water solubility. The overall effect results into low bioavailability and poor biological activity. Solubility is an important parameter which includes the dissolution, absorption and bioavailability of drug. The potential effects of a drug get reduced due to its low solubility. For solubility enhancement various approaches are reported including solid dispersions, cyclodextrin complex formation or chemical modifications. But various limitations are associated with these approaches [11]. Application of nanoparticle system is one of the approaches established to overcome these limitations. Various biodegradable nanoparticle formulations were reported for achieving increased drug solubility, targeted drug delivery, increased blood circulating time and prolonging of the biological activity [12].

Poly (D, L-lactide-co-glycolide) (PLGA) have been widely used for development of nano particle systems [13]. Folic acid is a water soluble vitamin which is oxidized form of folate. It is used as a ligand because of its efficient internalization capacity via folate receptor mediated endocytosis for tumor targeting. Folic acid is abundantly expressed in various types of human tumors such as breast, ovarian and prostate cancer. In normal tissues it is minimally distributed. Therefore, Folic acid can serve as a functional tumor-specific receptor and potential tumor marker [14-16].

Some attempts based on nanotechnology have been reported for Indian propolis as therapeutic agent including drug delivery system as anticancer agents [17,18]. However effects of formulation variables, detailed characterization of formulations, *in vitro* and *in vivo* cytotoxicity performance have not been studied. In addition, natural products are known for their activities through synergistic mechanisms where designing a delivery system by incorporating the optimized and standardized extract would be worthwhile compare to single drug formulation. So prompted by the above facts, the present study has been designed to develop standardized ethanolic extract of Indian propolis (EEIP) loaded PLGA– folic acid conjugated polymeric nanoparticles (denoted as ELFPN) using Design of Experiment approach to solve issue of poor water solubility, study synergized anticancer potency of EEIP, achieve sustained drug release and enhanced *in vitro* and *in vivo* anticancer efficacy.

Materials

PLGA with a copolymer ratio of d-l-lactide to glycolide of 50:50 (Mw 40,000–100,000 g/mol),1,3,Dicyclo-hexyl carbodiimide (DCC), N-hydroxysulfosuccinamide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), dialysis bag with a 12,000 Da molecular weight cut off, poly(vinyl alcohol) PVA (87–89% hydrolysis degree with molecular mass 12,000–13,000 g/mol were purchased from Sigma-Aldrich Bangalore, India. Acetone GR grade, sodium hydroxide, potassium dihydrogen phosphate, dichoromethane (DCM) and ethanol were purchased from Merck India. All other analytical grade chemical reagents were used.

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Methods

Ethanolic extract of Indian propolis (EEIP)

EEIP was prepared by extraction of crude propolis (10 g) pretreated with hexane and ethyl acetate to remove wax. Further extraction was done with ethanol by soxhlet method at 60°C to obtain EEIP. The obtained EEIP was standardized for content of CAPE using developed reverse phased high performance liquid chromatography (RP-HPLC) method and stored in 2-8°C until used for further evaluation.

Preparation of polymeric nanoparticles

EEIP loaded PLGA nanoparticles was prepared following oil in water (o/w) single emulsion solvent evaporation method described by Song (2008) with minor modifications. Obtained nanoparticles were designated as ENP. 3^2 factorial design was followed as per composition shown in Table 1 for batch optimization. Amount of EEIP (10mg) was kept constant for all batches. PLGA and EEIP were dissolved individually in dichloromethane (DCM) and ethanol respectively. These solutions were mixed and stirred well. This organic solution was emulsified with the 5 mL aqueous solution of PVA 1 by sonication (output power 70W, power of 80%, and 2 minutes) using a probe sonicator (U 200 S Control, Ika labrortech staufen, Germany) over an ice bath. The o/w single emulsions were stirred overnight under magnetic stirring at 1500 rpm at room temperature until complete evaporation of organic phase. The resulting particles were collected by centrifugation at 9000× rpm for 40 min (eppendorf Centrifuge 5424 R). Collected nanoparticles washed three times with ultra-pure water to remove excess PVA and separated from the free active ingredients or polymer agglomerate by filtration through a 0.45 µm membrane filter and then lyophilized. Lyophilized nanoparticles were stored at 4°C until used [19].

Nanoparticle characterization

Laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK) was used for Particle size (PS) determination. The PS measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water. Average PS was determined, expressed in terms of d (0.9) µm.

The concentration of EEIP (Encapsulation efficiency (EE) and Drug content (DC)) in the polymeric nanoparticle formulations was measured by HPLC method. A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 mL/min using BDS Hypersil C_{18} (250 mm × 4.6 mm; 5 µ particle size) Thermo scientific column was found to give desirable separation. Injection volume used was 10 µL, and the detection wavelength was set at 331 nm. Temperature was maintained at 25°C ± 2°C. The nanoparticle solution was suitably diluted with alcohol prior to determination.

The percent DC was calculated according to the equation:

DC (%) = (Amount of EEIP in nanoparticle/ Amount of EEIP and polymer) × 100 (1)

The unencapsulated EEIP was separated from nanoparticle suspension by centrifugation (eppendorf Centrifuge 5424 R) at 12,000 rpm for 2 h at 4°C and EE was determined. Sedimented nanoparticles were disrupted using acetone to release the entrapped EEIP which was suitably diluted with phosphate buffer pH 7.4 and analyzed by developed HPLC method (Jasco UV 2057, Japan). The percent EE was calculated using Equation (2).

EE (%) = Amount of EEIP entrapped in the vesicle/ Initial amount of the EEIP added × 100 (2)

Design of experiments DoE approach

Optimization of nanoparticle formulation was done by DoE approach. Particle size (PS) and encapsulation efficiency (EE) were studied for all batches. For the statistical design, Design Expert® Version 10.0 was used. EE and PS were selected as dependent variables while the amount of PLGA and amount of PVA were selected as independent variables.

Optimization by 3^2 factorial design

Based on preliminary experimentation data variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles were understood. The amount of polymer (X1) and surfactant (X2) were identified as crucial factors in determining the properties of the drug loaded polymeric nanoparticles. For optimization of the nanoparticles composition, 3^2 factorial design was adopted. Effect of X1 and X2 (independent variables) on PS and EE (two dependent variables or responses) was studied with 3D response surface plot.

Surface conjugation with folic acid

The surface modification was performed using method described by Das and Sahoo (2012) with minor modification. A composition of Folic acid: DCC: NHS = 1:1.2:2 in dimethyl sulphoxide (DMSO) was reacted for 6 hrs at 50°C. Removal of N, N- dicyclohexylurea (DCU) formed in reaction was done by filtration. Formed product was allowed to react over night with ethylene diamine using pyridine which acts as catalyst. The aminated folate formed in reaction was precipitated out with addition of excess cold acetonitrile which is separated by vacuum filtration. For conjugation of aminated folate onto the surface of optimized ENP, 20 mg of ENP was dispersed in 10 mL of phosphate buffer solution (PBS) (0.02 M, pH 7.4). 250 µl of EDC (2 mg/mL) and NHS (2 mg/mL) each were added and allowed to agitation for 2 hrs at room temperature under magnetic stirring. Unreacted EDC and NHS were removed by ultracentrifugation at 20,000 rpm for 20 min at 4°C (eppendorf Centrifuge 5424 R). Activated nanoparticles were recovered. For final conjugation, activated nanoparticles were dispersed in 4 mL of PBS (0.02 M, pH 7.4). 200 µl of aminated folate solution (1 mg/mL in PBS) was added. Solution was kept under magnetic stirring for 2 hrs at room temperature and unconjugated folate was removed by ultracentrifugation and lyophilized for further use. Fourier transform-infrared spectroscopy (FTIR) analysis was done for confirmation of conjugation [20].

Differential scanning calorimetry (DSC) and Fourier transform-infrared spectroscopy (FTIR)

Vacuum evaporated samples (EEIP and ELFPN) were analyzed by DSC using DSC 821e (Mettler-Toledo, Greifensee, Switzerland). Samples (5 mg) were hermetically sealed in aluminium pan and heated at heating rate of 10°C/min under a nitrogen atmosphere (flow rate 50 mL/min).

KBr dispersion method was used for FTIR study. FTIR spectra of EEIP, PLGA, Folic acid and ELFPN were recorded on a FTIR spectrophotometer (JASCO FTIR-8400, Japan). All samples were scanned in the range of 4000–400 cm^{-1}.

Surface morphology study

Surface morphology of nanoparticle suspensions was studied at magnification of 50000× using Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV. On carbon film-covered copper grid a drop of ENP and ELFPN suspension was applied. Thin film was formed by blotting extra suspension using filter paper. Sample was examined and photographed.

Zeta potential

Laser doppler electrophoretic mobility technique was used for measurements of zeta potential using Zetasizer 3000 (Malvern Instruments, UK) at a temperature of 250°C.

In vitro drug release study:

Dialysis bag diffusion technique was used for *in vitro* release of EEIP and ELFPN from the nanoparticle formulations in phosphate-buffer saline (PBS) (pH 7.4). ELFPN equivalent to 2 mg of EEIP was placed into a dialysis bag (cut off 12,000D).The bag was immersed into 50 mL of release medium in a beaker under magnetic stirring at 100 rpm at 37 + 0.5°C. Sample (5 mL) was withdrawn at predetermined time intervals (0.0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48 and 54 h) by maintaining sink condition. Pure EEIP release was analyzed as control in a similar manner by taking 2 mg EEIP solution (2 mg/mL in 50% w/w mixture of PEG 400 and water). The samples were analyzed in triplicate for EEIP content by developed HPLC method, (Jasco UV 2057, Japan). The percent cumulative release of EEIP was plotted against time [21].

In vitro cytotoxicity studies

Cytotoxicity study of EEIP and ELFPN on human breast cancer cell line MCF-7 was done by Sulforhodamine B (SRB) assay technique. ELFPN was diluted with distilled water to obtain EEIP in the concentration ranges of 10, 20, 40 and 80µg/mL. MCF-7 cells were cultured in RPMI1640 medium, 2 mM L-glutamate and 10% v/v fetal bovine serum (FBS) was supplemented. 96 well plate method was used where 5×10^3 cells per well was seeded. Trichloroacetic acid (TCA) was used as in-situ fixing agent. Further cells were incubated at 37°C with 100% relative humidity for 24 hrs. After incubation growth medium was replaced with 100 µL of fresh medium containing various concentrations (10-80 µg/mL) of EEIP and ELFPN. For control, culture media without any drug or formulation was used. Further after 48 hrs incubation, assay was terminated by adding 50 µL of the cold TCA and again incubated for 60 min at 4°C. Media was removed and washed with sterile PBS and air dried. In each well 50 µL of SRB solution (0.4% w/v in 1% acetic acid) was added and incubated for 20 min at room temperature. Washing with 1% acetic acid was done for removal of unbound dye and plates were allowed to air dry. 10 mM trizma base was used to elute bound stain and absorbance was measured using ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated using the following equation.

Cell growth (%) = (Absorbance (Average) of the test well / Absorbance (Average) of the control wells) ×100 [22].

Hemolysis study

The hemolytic study on the EEIP and ELFPN was done as per method described by love et al. (2012). 10 ml of fresh blood was collected and fibrinogen was removed. Further blood was diluted with aqueous NaCl solution (0.9% w/v) and centrifuged at 2000 rpm for 15 min. Supernatant was removed and the precipitate was diluted (1:50 v/v) with aqueous NaCl solution to obtain 2% red blood cell suspension. Tubes were labeled from 1–7 for each sample and each number contains a set of three tubes. 2.5 mL of the red blood cell suspension was added to all the tubes. In tube labeled as 1 which was taken as the hemolysis control (100% hemolysis), 2.5 mL of distilled water was added. In tube labeled as 2 which was taken as the non-hemolysis control (0% hemolysis), 2.5 mL of 0.9% isotonic NaCl solution was added. The remaining tubes were diluted with EEIP and ELFPN samples with concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mg/mL and made up to 5 mL with 0.9% isotonic NaCl solution. All tubes were allowed to incubate at 37°C for 1 h and then for kept 5 min at 0°C to stop hemolysis. Tubes were centrifuged at 2000 rpm for 15 min. Absorbance of supernatants was determined at 453 nm with UV–VIS spectrophotometer [23].

In vivo anticancer study

Male Swiss albino mice (20-25 gm) were used. They were acclimatized to laboratory environment at (temperature 25 ± 2°C) and 12 h dark /light cycle. Diet and water was supplied. Dalton's Lymphoma Ascites (DLA) cells were supplied by Amla cancer research center, Trissur, Kerala, India. Cell counts done and further dilutions were made to total cell count of 1×10^6. Before starting treatments, tumor growth in the mice was allowed for minimum seven days.

Animals were divided in to nine groups of six each. G2- G9 was injected with DLA cells (1×10^6 cells per mouse) intraperitonially (i.p.). Group 1(G1) and Group 2 (G2) were taken as the normal control and tumor control respectively. Group 1 and 2 supplied with normal diet and Water. Group 3 (G3) was taken as the positive control and was treated with injection 5-fluorouracil at 20 mg/kg body weight i.p. Group 4 (G4), Group 5 (G5) and Group 6 (G6) were administered with EEIP at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight i.p. respectively. Group 7 (G7), Group 8 (G8) and Group 9 (G9) were administered ELFPN at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight i.p respectively.

After 24 h of inoculation, treatment was given once daily for 14 days. After treatment all animals from each group were sacrificed. Retro orbital plexus bleeding method was used for blood withdrawal. Hematological parameters which include RBC count, WBC count, platelet count, Hb content and packed cell volume; serum enzyme level, lipid profile which include alkaline phosphatase (ALP), total cholesterol (TC), aspartate amino transferase (AST), triglycerides (TG), and alanine amino transferase (ALT) and derived parameters - life span (%), body weight, and cancer cell count were evaluated [24-26].

Stability study

ELFPN was transferred in glass vial and kept for short term stability study at 4°C in refrigerated condition. Optimized nanoparticles were analyzed for the change in PS and EE up to 180 days.

Statistical analysis

The results were expressed as mean ± S.D. One way ANOVA followed by Newman-Keul's multiple comparison test was used for evaluation of the data; $P<0.01$ implied significance.

Results and Discussion

Propolis is a natural bee hive product which majorly contains

various polyphenols and flavonoids and reported to possess potential antitumor effect on breast cancer cells. CAPE is one of the most potential constituent of propolis, however the isolation and purification process to obtain pure CAPE and some other chemical constituents is tedious, time consuming and costly. Moreover the synergistic effects of diverse chemical constituents of alternative medicines are well known compared to pure isolated compounds. So in present study attempt has been made to develop and compare nanoparticulate formulations of EEIP by considering its safety and anticancer efficacy on breast cancer.

In this study, EEIP loaded polymeric nanoparticles have been developed and investigated as injectable nano carriers in order to improve solubility, *in vitro* and *in vivo* anticancer efficacy. The effect of the ELFPN composition on PS and EE was studied by design of experiments (DoE) approach.

Effect of EEIP: PLGA on particle size and encapsulation efficiency was studied at different ratios (1:2.5/5/7.5/10). At 1:5 ratio minimum PS and maximum EE was observed. Further EEIP: PLGA was studied at ratios of (1:3.75/5/6.25). Results obtained were satisfactory to the requirement. Further detailed studies considering surfactant concentrations, dependant and independent variables were done for final optimization of formulation batch.

Nanoparticle characterization

Mean ENP nanoparticles size was in the range of 178 ± 5 to 205 ± 5 nm and was strongly affected by the selected variables. The DC and EE was in the range of 90.76-94.99% and 73.16–76.37 % respectively (Table 1). A good fit (r^2 for PS = 0.9971 and EE = 0.9809) was observed for the PS and EE the independent variables.

Optimization of ENP nanoparticles by 3^2 factorial design

PS and EE are important parameters considered in nanoparticle formulation development. During the preliminary study, polymers and surfactants concentrations were assessed for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, 3^2 factorial design was employed to optimize final proportions of PLGA and PVA (Table 1). The responses of these batches are shown in Table 1. Multiple regression analysis was done using Design Expert® Version 10.0. Results of the obtained data from multiple regression analysis are summarized in Table 2A.

From the factorial design study of ENP (Table 2B), it was observed that positive coefficients of the main terms X1 and X2 for EE and negative coefficients of the main terms X1 and X2 for PS indicated a favorable effect on the mean particle size and EE with the PLGA and PVA. The PLGA and PVA had a linear effect on EE and curvilinear effect on PS as seen in response surface plot (Figure 1). When amount of PLGA is low, smaller particles were obtained, which may be because of internal phase gets highly distributed into external phase. Further viscosity of internal phase gets increased with increased amount of PLGA which cause resistance for mass transfer from internal phase into the external phase during diffusion which leads of enlargement of particle size. Based on the results of the factorial design, the solution for optimum batch selection with highest desirability of 0.941 was obtained with F1 for ENP having acceptable PS and EE, so F1 for ENP was selected as an optimized formulation and further evaluated for various parameters.

Differential scanning calorimetry

For determination of molecular state of the EEIP, DSC study was performed for the free EEIP and ELFPN (Figure 2). In case of EEIP due to various polyphenols and flavonoids various melting transitions were observed. A sharp melting transition of was also observed at 127.02 °C with \H 17.59 J/g. In ELFPN thermogram, the free EEIP peaks were disappeared indicating molecular dispersion of EEIP inside polymeric nanoparticles.

Fourier transform-infrared spectroscopy (FTIR)

Figure 3 shows FTIR spectra of pure EEIP (A), PLGA (B), Folic acid (C) and ELFPN (D). It can be seen from the FT-IR spectrum of free EEIP showed various peaks at 3571.52 cm^{-1}, 3541.63 cm^{-1}, 3485.7 cm^{-1} and 3333 cm^{-1} that were assigned to –OH stretching. Band obtained at 3083.62 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm^{-1}, 1594.8 cm^{-1}, and 1164.7 cm^{-1} were attributed to C=O, C=C, and C–O stretching, respectively. In the FTIR spectrum of PLGA the bands at 2995 cm^{-1} and 2949 cm^{-1} were due to C–H stretch of CH_2 and C–H stretch of –C–H–, respectively. A band at 1772 cm^{-1} and 1751 cm^{-1} was assigned to the stretching vibration of C=O of ester bond (strong and narrow) and 1186– 1087 cm^{-1} was attributed to C–O stretching, which belongs to the characteristic peaks of PLGA molecule.

However, in the FTIR spectra of ELFPN, the major peaks at 3571.52 cm^{-1}, 3541.63 cm^{-1} were assigned to –OH stretching. Band obtained at 3083.62 cm^{-1} was assigned to C–H stretch. The strong and narrow peaks at 1639.2 cm^{-1}, 1594.8 cm^{-1}, and 1164.7 cm^{-1} were significantly decreased by its intensity and the presence of these characteristic peaks is a confirmation of EEIP encapsulation on PLGA nanoparticles successfully.

Table 1: The effect of various formulations (with different amounts of surfactant and polymer) on PS and EE by 3^2 factorial design with coded levels and actual amount values of variables.

Batches ENP	Coded levels ($X1, X_2$)	Amt. of PLGA (mg; X_1)	Amt of PVA (mg; X_2)	Drug Content (%)	Particle size (nm; Y_1)	Encapsulation efficiency (%;Y_2)
F1	+1, +1	62.50	100.00	93.66 ± 0.77	178 ± 5	76.37 ± 1.89
F2	+1, 0	62.50	75.00	94.99 ± 1.55	180 ± 4	76.20 ± 1.96
F3	+1,-1	62.50	50.00	93.13 ± 1.25	183 ± 4	75.83 ± 1.44
F4	0, +1	50.00	100.00	93.12 ± 1.40	184 ± 4	75.42 ± 1.44
F5	0, 0	50.00	75.00	92.11 ± 1.00	185 ± 5	74.61 ± 1.58
F6	0, -1	50.00	50.00	91.84 ± 1.25	191 ± 4	74.18 ± 1.44
F7	-1, +1	37.50	100.00	91.42 ± 1.25	199 ± 4	74.16 ± 1.96
F8	-1, 0	37.50	75.00	90.82 ± 0.98	200 ± 5	73.62 ± 1.89
F9	-1, -1	37.50	50.00	90.76 ± 0.98	205 ± 5	73.16 ± 1.89

Values were presented as mean ± SD (n=3).

Table 2A: The results of multiple regression analysis of the obtained data.

Sr. no	Final Equation in Terms of Coded Factors		
	EE	PS	
1	+74.38	+190.22	
2	+4.97	-43.00	* X1
3	+0.93	-12.67	* X2
4	-	+2.00	* X1X2
5	-	+66.67	* $X1^2$
6	-	+6.67	* $X2^2$

Table 2B: Optimized batch selection with desirability index.

Number	PLGA	PVA	EE	PS	Desirability
ENP	62.500	100.000	76.546	178.139	0.941

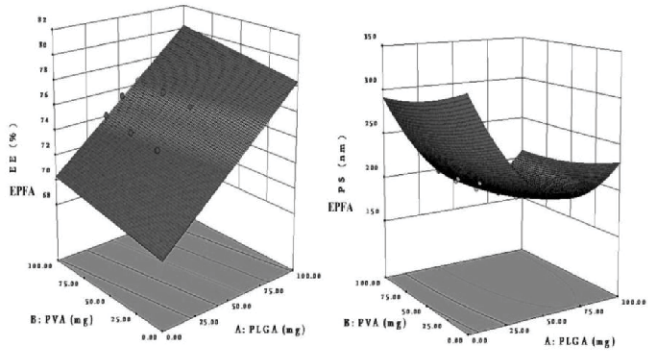

Figure 1: Response surface plot showing effects of amount of PLGA and PVA (independent variables) on dependent variables PS and EE for ELFPN.

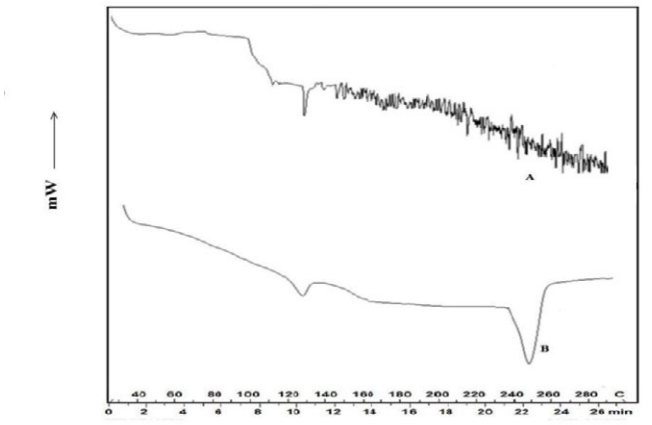

Figure 2: Comparative DSC thermo grams of EEIP (A) and ELFPN (B).

Conjugation of folic acid to PLGA nanoparticles was confirmed by the amide bond peak at 1634.38 cm-1 in ELFPN.

Surface morphology study

Surface morphology of the ENP and ELFPN was assessed using transmission electron microscope (TEM). As observed in Figure 4, it can be seen that the nanoparticles were freely dispersed and spherical in shape. In comparison with ENP, ELFPN have slight larger particle size. This may be due to surface modification by conjugation of folic acid.

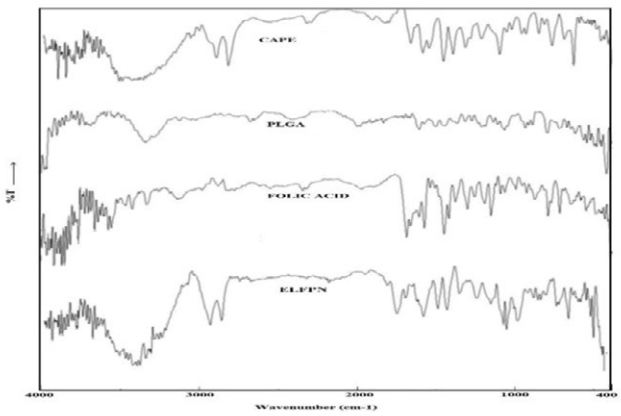

Figure 3: Comparative FTIR spectra of pure EEIP, PLGA, Folic acid and ELFPN.

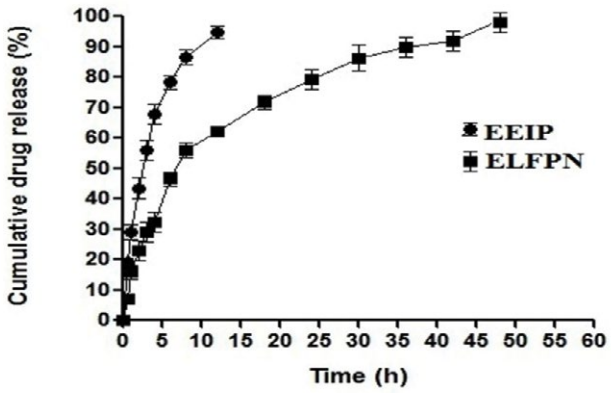

Figure 4: Transmission electron microscopy images of ENP (A) and ELFPN (B).

Zeta potential

Zeta potential is important parameter that gives information about surface charges which has direct impact on colloidal stability and interaction with physiological body cells. ENP showed negative zeta potential –17.71 ± 0.51 mV which may be due to presence of polyvinyl alcohol on surface. ELFPN showed slight shifting of zeta potential towards positive (–13.11 ± 0.88 mV) which may be due to amide bond of folic acid after surface modification. In the present study, it seems to be in appropriate values of zeta potential considering the colloidal stability of nanoparticles.

In vitro release study

In vitro release of the EEIP from ELFPN was investigated by diffusion bag technique using phosphate buffer saline (pH 7.4) as a release medium at 37°C. Figure 5 revealed that EEIP could freely diffuse within 6h in its solution form causing 96.79% drug release. However, EEIP release from ELFPN showed a biphasic pattern with initial burst release (23.78 % respectively) within the first 1 h followed by sustained release up to 48 h for ELFPN.

Erosion, diffusion and degradation are types of basic mechanisms contributed for the release of a drug from polymeric nanoparticles. Any one or all three mechanisms may be associated with drug release for developed formulation. Initial burst release occur may be due to the presence of drug dissolved in medium or adsorbed on the surface of the nanoparticles, while a sustained release could be caused by diffusion of the drug. For development of desired formulation, sustained release of entrapped drug from nanoparticles is an important parameter, as it maintains constant amount of drug persistently at site of action. As shown in Figure 5 the release profile of EEIP from nanoparticulate system exhibited a biphasic drug release pattern that was characterized by an initial rapid release followed by a slower continuous release phase over 48 h.

In vitro anticancer activity

The *in vitro* anticancer activity of ELFPN was studied in comparison with EEIP against human breast cancer MCF-7 cells using *in vitro* SRB assay. The results illustrated in Table 3 and Figure 6 indicates that ELFPN showed better cytotoxicity than EEIP. The total growth inhibition concentration of ELFPN and EEIP was found to be 17.0±0.010 µg/mL and 30.10±0.015 µg/mL respectively. TGI value of ELFPN was found to be 43.34% less than the EEIP.

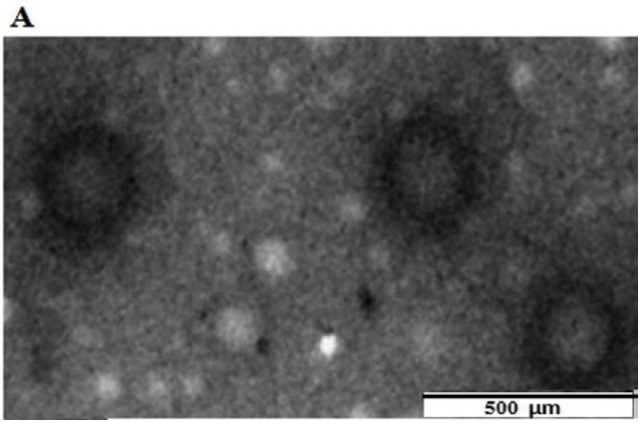

Figure 5: *In vitro* release of pure EEIP and EEIP from ELFPN.

The enhanced anticancer efficacy may be attributed to greater cellular uptake of ELFPN via phagocytosis or the fusion process. Greater cellular uptake may results in the enhanced permeability of the cell membrane to EEIP that allows sufficient drug concentration inside the cells. Therefore, ELFPN might have served as a potential nano-carrier to improve *in vitro* anticancer activity of EEIP. The lower anticancer activity of free EEIP may be due to its efflux by P-glycoprotein pumps. The observed GI50 values for EEIP and ELFPN was less than 10 µg/mL indicate potent anticancer nature.

Hemolysis study

It is necessary to study the biosafety by hemolysis study for *in vivo* application of developed formulation. The hemolytic potential was evaluated using optical density method. The result indicates that the hemolysis rate of EEIP and ELFPN was 2.8 and 2.6 % respectively up to 1.5 mg/mL concentration. The acceptable hemolysis rate (less than 3%) [21,27] was observed for EEIP and ELFPN having non-hemolytic

Table 3: TGI and GI_{50} values of EEIP, ELFPN and ADR on MCF-7 breast cancer cell line.

Samples	MCF-7 cell line TGI (µg/mL)	MCF-7 cell line GI_{50} (µg/mL)
EEIP	30.10 ± 0.015	<10
ELFPN	17.0 ± 0.010	<10
ADR	<10	<10

Values were presented as mean ± SD (n=3).
TGI- Concentration of drug that produce total inhibition of cells, GI50- Concentration of drug that produce 50% inhibition of cells, MCF 7- cell line.

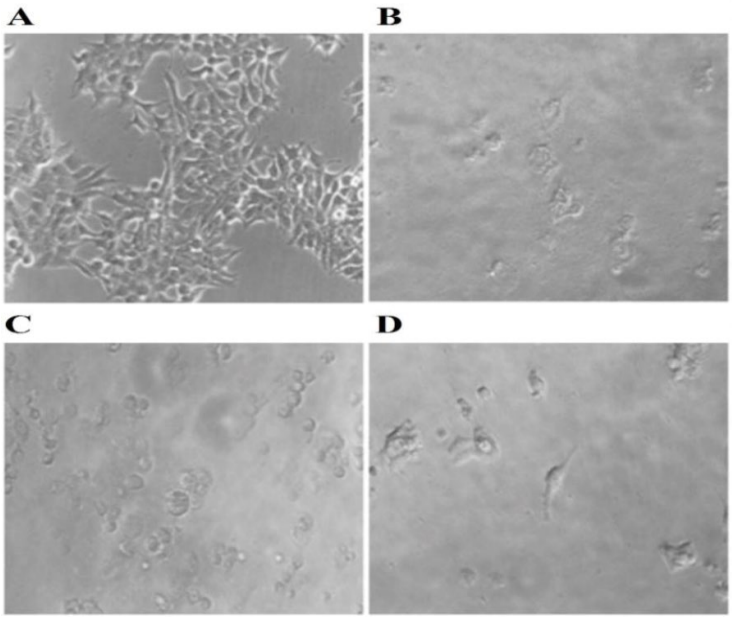

Figure 6: *In vitro* cytotoxicity study on MCF-7 Cell lines (A) MCF-7 control (B) Positive control ADR (C) EEIP (D) ELFPN Treated.

property up to 1.5 mg/mL. Considering the obtained results ELFPN may be considered as biosafe for internal use.

(i) *In vivo* anticancer study

As shown in Table 4 the average life span in DLA tumor control group was found to be 48%. In case of animals treated with EEIP at a dose of 100,200,400 mg/kg, increase in body weight and life span was observed, whereas ELFPN at a dose of 100,200,400 mg/kg body weight increased the life span to 93.5%, 94% and 94.5% respectively. The reason for increase in life span with ELFPN treated group may be because of increased solubility of EEIP when developed as nanoparticles formulation system. These values were significantly deviated from normal control (G1) at P<0.01 and cancer control (G2) at P<0.01. However the average life span of animals with 5-FU treatment group was found to be 96%.

Significant reduction in percent increase in body weight of animals when compared to DLA tumor bearing mice was also observed in group treated with ELFPN nanoparticle formulation at a dose of 100,200,400 mg/kg. Significant reduction in percent increase in body weight, significant reduction in packed cell volume and viable tumor cell count in animals compared to DLA tumor bearing mice after treatment with ELFPN indicate its antitumor nature (Table 4 and 5). In case of ELFPN more reduction in packed cell volume and viable Tumor cell count was observed that may be due to increased solubility of EEIP. Drug availability might have increased on site of action because of achieving EPR effect and greater cellular uptake due to reduction in particle size.

In DLA control group (Table 5) WBC count was significantly increased while Hb, RBC and platelets counts were decreased as compared to the normal control group. Treatment with ELFPN at a dose of 100,200,400 mg/kg significantly increases the RBC, Hb content, platelet counts and decreases WBC count to about normal level. From the results it can be concluded that the ELFPN nanoparticle formulation showed comparatively better effects on hematological parameters that showed the developed system is biocompatible with less hemolysis.

The inoculation of DLA cells caused significant increase in the level of Aspartate amino Transferase, Alkaline Phosphatase, total Cholesterol and Alanine amino Transferase in the tumor control animals (G2), when compared to the normal group. The treatment with ELFPN nanoparticle formulation reversed these changes towards the normal level (Table 6).

(ii) Stability studies

No significant deviations in EE and PS values were observed over the period of six months as compared to initial values (P>0.05) of freshly prepared nanoparticle formulation during stability study which indicates the developed formulation was physically stable for at least 6 months.

Conclusion

Folic acid conjugated nanoparticulate formulation composed of PLGA and PVA as an injectable nano carrier for the delivery of a

Table 4: Effect of ELFPN on the life span, body weight and cancer cell count of tumor induced mice.

Treatment Groups	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G1	6	>>30 days	2.22 ± 0.68	-
G2	6	48%	9.44 ± 1.86^{a**}	2.75 ± 0.80^{a**}
G3	6	96%	5.66 ± 0.42^{b**}	1.30 ± 0.22^{b**}
G4	6	88%	5.45 ± 0.32^{b**}	1.42 ± 0.30^{b**}
G5	6	89%	5.36 ± 0.30^{b**}	1.40 ± 0.28^{b**}
G6	6	90%	5.30 ± 0.28^{b**}	1.38 ± 0.24^{b**}
G7	6	93.5%	4.94 ± 0.14^{b**}	1.32 ± 0.12^{b**}
G8	6	94%	4.92 ± 0.10^{b**}	1.31 ± 0.08^{b**}
G9	6	94.5%	4.90 ± 0.08^{b**}	1.30 ± 0.05^{b**}

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (ELFPN 100,200,400 mg/kg).
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from normal control (G_1) at P<0.01
**b – Values are significantly different from cancer control (G_2) at P<0.01

Table 5: Effect of ELFPN on Hematological parameters.

Treatment Groups	Total WBC Cells /mlx10^3	RBC Count millions/mm^3	Hb gm/dl	PCV %	Platelets Lakhs/ mm^3
G1	10.35 ± 1.05	4.55 ± 1.95	12.90 ± 1.95	14.25 ± 2.44	3.60 ± 0.95
G2	15.30 ± 2.60^{a**}	2.70 ± 0.98^{a**}	6.80 ± 0.95^{a**}	38.36 ± 3.35^{a**}	1.70 ± 0.42^{a**}
G3	12.30 ± 1.34^{b**}	4.05 ± 1.62^{b**}	11.90 ± 1.48^{b**}	16.40 ± 1.40^{b**}	2.94 ± 0.50^{b**}
G4	12.12 ± 1.26^{b**}	4.06 ± 1.50^{b**}	12.22 ± 1.52^{b**}	17.30 ± 2.36^{b**}	3.30 ± 0.65^{b**}
G5	12.05 ± 1.22^{b**}	4.08 ± 1.60^{b**}	12.25 ± 1.55^{b**}	17.24 ± 2.30^{b**}	3.36 ± 0.68^{b**}
G6	11.85 ± 1.18^{b**}	4.12 ± 1.65^{b**}	12.35 ± 1.60^{b**}	17.20 ± 2.26^{b**}	3.40 ± 0.70^{b**}
G7	11.48 ± 0.95^{b**}	4.35 ± 1.89^{b**}	12.55 ± 1.72^{b**}	16.64 ± 1.88^{b**}	3.58 ± 0.85^{b**}
G8	11.44 ± 0.90^{b**}	4.38 ± 1.92^{b**}	12.58 ± 1.75^{b**}	16.60 ± 1.84^{b**}	3.62 ± 0.88^{b**}
G9	11.40 ± 0.88^{b**}	4.40 ± 1.95^{b**}	12.62 ± 1.78^{b**}	16.56 ± 1.80^{b**}	3.65 ± 0.92^{b**}

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (ELFPN 100,200,400 mg/kg)
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from normal control (G_1) at P<0.01
**b – Values are significantly different from cancer control (G_2) at P<0.01

Table 6: Effect of ELFPN on serum Enzymes and lipid proteins.

Treatment Groups	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G1	108.85 ± 3.05	136.85 ± 2.55	36.40 ± 1.65	31.28 ± 1.45	132.28 ± 2.08
G2	146.95 ± 4.34^{a**}	220.28 ± 4.40^{a**}	78.6 ± 2.94^{a**}	62.32 ± 2.60^{a**}	265.30 ± 4.35^{a**}
G3	126.30 ± 3.84^{b**}	169.15 ± 2.65^{b**}	44.40 ± 1.72^{b**}	34.52 ± 1.70^{b**}	154.45 ± 2.40^{b**}
G4	117.26 ± 3.42^{b**}	160.08 ± 2.55^{b**}	42.44 ± 2.30^{b**}	35.28 ± 1.55^{b**}	162.45 ± 2.22^{b**}
G5	115.18 ± 3.38^{b**}	156.25 ± 2.50^{b**}	41.60 ± 2.20^{b**}	34.90 ± 1.42^{b**}	160.48 ± 2.18^{b**}
G6	113.36 ± 3.26^{b**}	153.30 ± 2.46^{b**}	40.90 ± 2.16^{b**}	34.80 ± 1.38^{b**}	158.45 ± 2.15^{b**}
G7	110.05 ± 3.02^{b**}	143.65 ± 2.26^{b**}	39.42 ± 1.95^{b**}	33.35 ± 0.92^{b**}	151.30 ± 1.95^{b**}
G8	109.12 ± 2.95^{b**}	142.62 ± 2.20^{b**}	39.32 ± 1.90^{b**}	33.22 ± 0.85^{b**}	150.34 ± 1.88^{b**}
G9	109.20 ± 2.90^{b**}	142.25 ± 2.18^{b**}	39.22 ± 1.85^{b**}	33.05 ± 0.80^{b**}	150.30 ± 1.85^{b**}

G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 toG6–Treatment control (EEIP 100,200,400 mg/kg), G10 toG12 Treatment control (ELFPN 100,200,400 mg/kg)
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from normal control (G_1) at P<0.01
**b – Values are significantly different from cancer control (G_2) at P<0.01

natural anticancer bioactive, EEIP were investigated. The developed formulation showed smaller particle size, excellent encapsulation efficiency and control release with better stability. ELFPN displayed better cytotoxicity in terms of TGI as compared to EEIP. ELFPN demonstrated higher *in vitro* cytotoxicity as well as significant *in vivo* anticancer activity than its free form which may leads to reduction in dose. Developed formulation can be considered as a novel dosage form for EEIP with enhanced therapeutic efficacy in cancer chemotherapy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Original Research Article (Experimental)

Standardization, anti-carcinogenic potential and biosafety of Indian propolis

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ABSTRACT

Background: Propolis from apiculture is known for wide range of medicinal properties owing to its vast chemical constituents including polyphenols, flavonoids and anticancer agent Caffeic acid phenethyl ester (CAPE).
Objectives: The objective of the study was to extract and standardize Indian propolis (IP) with respect to selected markers by newly developed High performance liquid chromatography (HPLC) method, to evaluate *in vitro* and *in vivo* anticancer activity and biosafety of Indian propolis.
Materials and methods: IP was extracted, optimized and standardized using a newly developed and validated HPLC method for simultaneous estimation of caffeic acid, apigenin, quercetin and CAPE. The standardised ethanolic extract of IP (EEIP) was screened for *in vitro* cytotoxicity using sulforhodamine B (SRB) assay, *in vivo* anti-carcinogenic effect against Dalton's Lymphoma ascites (DLA) cells, hemolytic effect and pesticide analysis.
Results: The EEIP was found to contain more amount of total flavonoids (23.61 ± 0.0452 mg equivalent of quercetin/g), total polyphenolics (34.82 ± 0.0785 mg equivalent of gallic acid/g) and all selected markers except caffeic acid compared to all other extracts. EEIP showed better anti-cancer potential than CAPE on MCF-7 and HT-29 cell line and significant ($p < 0.01$) *in vivo* anti-carcinogenic effects against DLA in comparison with 5-fluorouracil. EEIP was found to be non-hemolytic.
Conclusion: From *in vitro* cytotoxicity, *in vivo* anti-carcinogenicity and biosafety studies it can be concluded that the standardized EEIP is safe and can be considered for further development as a biomedicine.

1. Introduction

Natural products have proved to be a rich source of various constituents that have found to possess antitumor activity and applications in cancer chemotherapy [1]. One such natural product, propolis is an important healthy food ingredient which has nutritional and medicinal properties obtained from apiculture [2,3]. Physical appearance of propolis depends upon plants from which this resinous substance is collected. Generally propolis is yellowish brown to almost black in color. Propolis is naturally available in resinous and wax form. It is a sticky and gummy form derived from honey bees used as building and insulating material to honey combs. Its smell is pleasant due to honey, wax and vanilla but has a bitter taste. Its melting point is usually 60–70 °C [4]. The various complex chemical constituents possessed by propolis varies according to geographical origin and depends greatly upon bee-released and plant-derived compounds. Generally raw propolis contains resins (50%), waxes (30%), essential oils (10%), pollen (5%), and various organic compounds (5%). Till now, more than 300 different constituents were reported from propolis. Composition of propolis also varies depending upon collection time and place [5]. Propolis has been widely used for its nutritional and medicinal values since ancient time in Greece, Roman empire, Egypt and various countries. Even today it continues to be a popular remedy, as natural product and as a healthy food [6]. Stingless bees belong to *Apidae* family and these are exclusively observed in tropical and subtropical regions. They act as pollinators. They collect pollens and nectar from various medicinal plants which include Coco, palm, tulsi etc. [7]. Indian propolis is available throughout the India and

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according to different geographical origin its chemical composition varies. Various studies are reported on Indian propolis of different geographical region which includes Karnataka, West Bengal, Gujarat, Madhya Pradesh, Maharashtra and Rajasthan [5].

Apart from the use of propolis as a nutritional and healthy food constituent, its use as folk medicine in human health [8] has been reported from ancient time. Propolis of various regions is known to exhibit various activities including anti-fungal, antibacterial and anti-cancer activity [9–11]. Use of propolis as an anti-inflammatory, anti-fungal, anti-viral, anti-ulcer has been reported in Ayurveda, Homeopathy and acupuncture [12]. In Egypt, traditionally propolis was used as an anti-putrefactive and anti-pyretic agent. Greeks and Romans used propolis as a mouth disinfectant, antiseptic and wound healing agent. Therapeutic use of propolis was also continued by Arab physicians until Middle Age. London pharmacopoeia listed propolis as official drug in 17th century and it became popular as an antibacterial agent in Europe in 17th and 20th centuries. In World War II, propolis was employed for tuberculosis treatment. Apart from this propolis was also reported in treatment of wounds, healing, burns, sore throat and stomach ulcer. First scientific work related to chemical properties and composition of propolis was published and indexed to chemical abstracts in 1908 [13].

Various *in vitro* and *in vivo* studies on propolis and its isolated constituents [14] have been reported for anti-cancer activity. The anti-cancer activity of propolis is attributed to Caffeic acid phenethyl ester (CAPE) in addition to polyphenols and flavonoids [15,16] CAPE is a specific inhibitor of NF-κB [16]. But isolation, purification or synthesis of CAPE is costly and tedious process. Various analytical methods have been reported for identification, separation of chemical constituents and standardization of Indian propolis extracts including HPLC, HPTLC and GC–MS. Chemical analysis methods have been also reported for quantification of polyphenols, flavonoids etc. [5,17–19].

Raw propolis cannot be used for delivery due to its complex structure. Various solvents have been reported for commercial extraction which includes water, methanol, ethanol, dichloromethane, ether etc. These solvent systems are used for removal of inert material as well as extract specific compounds. Biological activity varies depending upon extraction of solvent and method used [13,20–23].

Although propolis is extensively studied globally, the research on Indian propolis is at infancy. There is lack of studies on extraction optimization, analytical method and biosafety study on Indian propolis except few [7,18,24–27]. Hence, in the present study, an attempt has been made for extraction of Indian propolis by suitable method, standardization with respect to selected markers by newly developed reversed phase high performance liquid chromatography (RP-HPLC) method, evaluation of *in vitro* and *in vivo* anti-cancer activity to study synergistic effects of CAPE with other polyphenols and flavonoids and biosafety to explore Indian propolis as a biomedicine.

2. Materials and methods

2.1. Material

The Indian propolis sample collected in the month of December was purchased from local bee keeper from the Bharatpur region of Rajasthan, India and authenticated by Central Bee Research and Training Institute (CBRTI), Pune. Apigenin (> 99% purity) was purchased from Natural Remedies India Private Limited, Bangalore, India. Caffeic acid and CAPE (> 99% purity) were purchased from Sigma Aldrich, Bangalore, India. All reagents used were of analytical grade from Merck, India.

2.2. Total balsam content

1 g of crude propolis was accurately weighed and dissolved in 10 ml of ethanol, filtered and filtrate was evaporated to dryness until constant weight was obtained, and the ethanol soluble fraction was taken as percentage of balsam in the crude propolis sample [28].

2.3. Extraction and characterization of Indian propolis

2.3.1. Extraction

10 g of crude propolis was extracted with 30 ml of hexane by sonication for 30 min to remove the wax and filtered to obtain hexane extract of Indian propolis (HEIP). The mark was further extracted with ethyl acetate by sonication method and ethanol by Soxhlet method at 60 °C to obtain ethyl acetate extract of Indian propolis (EAEIP) and ethanolic extract of Indian propolis (EEIP) respectively. Further, mark was extracted with water by Soxhlet extraction at 100 °C to obtain water extract of Indian propolis (WEIP). All extracts were stored in 2–8 °C and used for further evaluation [24].

2.3.2. Total flavonoids and total polyphenol contents

Total flavonoids and polyphenol contents were determined by following methods reported by Marinova et al. and results were expressed in the form of mg equivalence of quercetin/g for flavonoids and mg equivalence of gallic acid/g for polyphenols [29–31].

2.4. Method development and validation

Individual stock solutions of caffeic acid, apigenin, quercetin and CAPE were prepared in ethanol to obtain 1 mg/mL solutions, diluted suitably to obtain working standards and stored in refrigerator (4 °C). 3 mg of extract was accurately weighed and dissolved in 1 ml of ethanol and diluted suitably to achieve concentration of 3000 μg/mL solution. HPLC separation was optimized using the aliquots of standard solutions ranging from 20–70 μg/mL for apigenin, caffeic acid and CAPE and 30–80 μg/mL for quercetin and analyzed. The proposed method was validated as per ICH guidelines (ICH Q2 (R1) 2005). Accuracy is determined by adding of known amount of analyte in the sample and expressed as % recovery. It was determined by calculating recovery of caffeic acid, apigenin, quercetin and CAPE by standard spiking method. For determination of intra-day and inter-day precision, solutions of 3 different concentrations were analyzed at 3 different time intervals in same day and different days and percent RSD was calculated.

Robustness of the method was determined by measuring the effect of small and deliberate changes in the analytical parameter on the retention time and peak area. The parameters selected were mobile phase concentration, flow rate and wavelength. While one parameter was altered remaining were kept constant. Standard deviation and percent standard deviation of peak area were calculated.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as per ICH guidelines based on standard deviation of the response and the slope. All extracts were standardized for caffeic acid, apigenin, quercetin and CAPE using developed HPLC method [32–35].

2.5. Total moisture content and pesticide analysis

Total moisture content of EEIP was determined using Mettle Toledo HB 43 Moisture analyzer. 3 g of raw propolis was kept on pan at 100 °C until constant weight was obtained.

Total pesticide content of EEIP was analyzed by Marco et al. using 410 Proster binary LC with 500 MS IT PDA detectors and EEIP was analyzed to check various types of pesticides [36].

2.6. In vitro anti-cancer study

In vitro anti-cancer study was carried out at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC, Navi Mumbai). The study was carried out by *in vitro* Sulforhodamine B assay method. The cell lines of MCF-7 (Human breast cancer) and HT-9 (Colon cancer) were procured from NCCS, Pune, India. The cytotoxicity study protocol for *in vitro* Sulforhodamine B assay was followed by method described by Bothiraja et al.

EEIP and CAPE were diluted in the concentration ranges of 10, 20, 40 and 80 μg/mL which were analyzed for cytotoxicity using SRB assay. The cells were cultured in RPMI 1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) and 2 mM L-glutamate. Cells were seeded at the density of 5×10^3 cells per well in 96-well plates using *in situ* fixing agent trichloroacetic acid (TCA). After 24 h of incubation at 37 °C with 100% relative humidity (RH), the growth medium was replaced with 100 μL of fresh medium containing various concentrations (10–80 μg/mL) of EEIP and CAPE. The culture medium without any drug formulation was used as a control. After 48 h incubation, assay was terminated by adding 50 μL of the cold TCA and incubated for 60 min at 4 °C. The medium was removed and washed with sterile PBS and dried. 50 μL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and further incubated for 20 min at room temperature. After staining, unbound dye was removed by washing with 1% acetic acid and plates were air dried. Bound stain was eluted with 10 mM trizma base and the absorbance was measured on an ELISA plate reader at a wavelength of 540 nm with 640 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test results relative to control wells using the following equation: [37].

$$\text{Cell growth } (\%) = (\text{Average absorbance of the test well} / \text{Average absorbance of the control wells}) \times 100$$

2.7. Hemolysis and plasma protein binding study

Hemolytic effect and the plasma protein binding ability of EEIP were evaluated using method described by Bothiraja et al. [37].

2.8. In vivo anti-cancer study

The study protocol was approved by Institutional Animal Ethics Committee of Bharati Vidyapeeth University, Poona college of Pharmacy, Pune, as per approval number CPCSEA/QA/06/2015-16. The study was carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Male Swiss albino mice (20–25 g) were used for the study. They were acclimatized to laboratory environment at temperature 25 ± 2 °C and 12 h dark/light cycle. Diet and water was supplied. Amla Cancer Research Center, Trissur, Kerala, India supplied the Dalton's Lymphoma ascites (DLA) cells. The cells were intraperitoneally (i.p) transplanted and maintained *in vivo* in Swiss albino mice. DLA cells were aspirated from peritoneal cavity of the mice using saline while transforming the tumor cells to the grouped animal. Cell counts were conducted and further dilutions were made so that total cell count should be 1×10^6. Before starting treatments, tumor growth in the mice was allowed for minimum seven days. Animals were divided in to six groups of six each. Group 1 (G1) and Group 2 (G2) were named as the normal control and tumor control respectively. G1 and G2 were supplied with normal diet and water. Group 3 (G3) served as the positive control which was treated with injection 5-fluorouracil at 20 mg/kg body weight, intraperitoneally. G2–G6 were injected with DLA cells (1×10^6 cells per mouse) intraperitoneally. Group 4 (G4), Group 5 (G5) and Group 6 (G6) were treated with EEIP at a dose of 100, 200 and 400 mg/kg intraperitoneally.

After 24 h of inoculation, treatment was given once daily for 14 days. After treatment, all animals from each group were sacrificed by euthanasia. Retro-orbital plexus bleeding method was used for blood withdrawal from each mouse. Hematological parameters like RBC count, WBC count, platelet count, Hb content and packed cell volume; serum enzyme and lipid profile which include alkaline phosphatase (ALP), total cholesterol (TC), aspartate amino transferase (AST), triglycerides (TG), and alanine amino transferase (ALT); and derived parameters like life span (%), body weight, and cancer cell count were evaluated [38–40].

2.9. Statistical analysis

The results are expressed as mean ± SD. One way ANOVA followed by Newman–Keuls multiple comparison test was used for evaluation of the *in vivo* study data; $p < 0.01$ implied significance.

3. Results

3.1. Characterization of Indian propolis sample

Preliminary analysis revealed that crude Indian propolis was yellowish brown in color, and sticky. It had a typical odor with a bitter taste. The pollens of *Brassica campestris*, *Eucalyptus species*,

Table 1
Summary of validation parameters.

Parameters	Caffeic acid	Quercetin	Apigenin	CAPE
Linearity range (μg/mL)	20–70	30–80	20–70	20–70
Regression equation	$y = 5024x + 1464$	$y = 37{,}619x - 79{,}941$	$y = 70{,}037x - 1{,}377{,}000$	$y = 56{,}240x - 60{,}582$
r^2	0.998	0.999	0.999	0.998
Slope	5024	37,619	70,037	56,240
Intercept	1464	79,941	1,377,000	60,582
Retention time (min)	1.993 ± 0.125	3.153 ± 0.073	3.757 ± 0.138	5.804 ± 0.069
Theoretical plates	2795 ± 0.088	3496 ± 0.078	5202.8 ± 0.898	7359.5 ± 0.188
LOD (μg/mL)	2.413	1.649	1.752	2.499
LOQ (μg/mL)	7.312	4.997	5.309	7.572
Precision				
Intra-day	≤ 2	≤ 2	≤ 2	≤ 2
Inter-day	≤ 2	≤ 2	≤ 2	≤ 2
Accuracy (%)	97.410–99.520	98.920–99.820	97.750–99.330	98.350–99.720
Robustness	≤ 2	≤ 2	≤ 2	≤ 2

r^2 – square of correlation coefficient, LOD – limit of detection, LOQ – limit of quantitation, μg/mL – microgram per milliliter, % RSD – percent relative standard deviation.

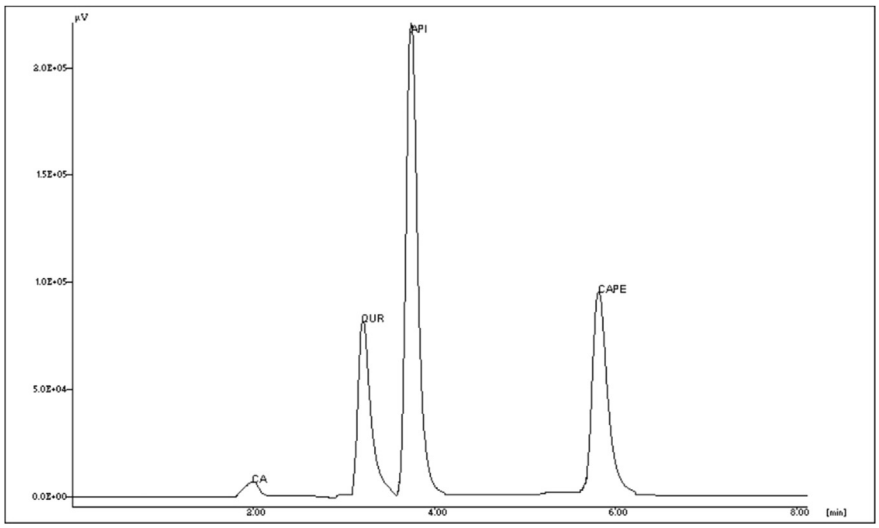

Fig. 1. RP-HPLC chromatogram of caffeic acid (CA), quercetin (QUR), apigenin (API) and caffeic acid phenethyl ester (CAPE).

Cocos nucifera, *Punica grantanum* and few grains belonging to *Asterace* family were present in Indian stingless bee propolis. Total balsam content was found to be 46% w/w. The total extraction yield of each extract was 18.23% w/w, 7.11% w/w, 49.00% w/w and 10.40% w/w for HEIP, EAEIP, EEIP and WEIP respectively. Total polyphenol content was found to be 18.06 ± 0.064 and 34.82 ± 0.078 mg equivalent of gallic acid/g in EAEIP and EEIP respectively. Flavonoid content of EEIP and EAEIP was found to be 23.61 ± 0.045 and 11.30 ± 0.011 mg equivalent of quercetin/g respectively.

3.2. Method development and validation

A mobile phase consisting of methanol and water (80:20 v/v) at flow rate of 1.0 mL/min using BDS Hypersil C_{18} (250 mm × 4.6 mm; 5 μ particle size) Thermo Scientific column was found to give desirable separation. Injection volume used was 10 μL, and the detection wavelength was set at 331 nm. Temperature was maintained at 25 °C ± 2 °C.

Acceptable response and detection of selected markers were obtained at wavelength 331 nm. Each run was followed by 10 min wash with methanol. Calibration curves were plotted which were found to ($n = 3$ repetitions of each point) be linear in the range of 20–70 μg/ml for caffeic acid, apigenin and CAPE, 30–80 μg/mL for quercetin and with good correlation co-efficient. Linear regression data, average retention time, LOD and LOQ for all markers is shown in Table 1. Representative chromatogram is shown in Fig. 1.

The percent relative standard deviation (% RSD) for intra-day and inter-day precision for all four markers was found to be less than ≤ 2. Satisfactory recoveries for all four markers were obtained as shown in Table 1. The robustness result showed that the peak areas remain unaffected (% RSD ≤ 2) which indicates that the proposed method is robust.

3.3. Standardization of extracts

The percent amount of apigenin, quercetin and CAPE in EEIP was found to be 1.005 ± 0.070, 1.344 ± 0.021 and 0.677 ± 0.002

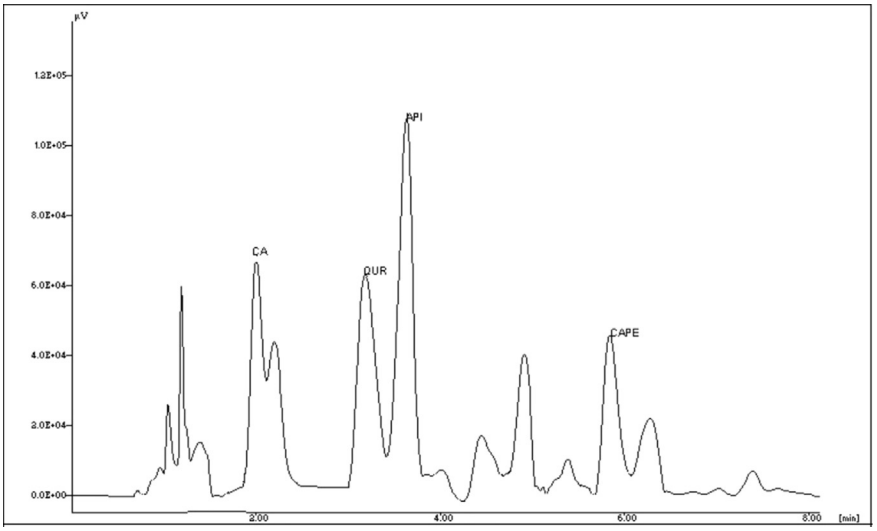

Fig. 2. RP-HPLC chromatogram of ethanolic extract of Indian propolis (EEIP) showing presence of caffeic acid (CA), quercetin (QUR), apigenin (API) and caffeic acid phenethyl ester (CAPE).

respectively. Amount of caffeic acid in WEIP was found to be 1.019% ± 0.016. Representative chromatograms are shown in Fig. 2.

3.4. Moisture content and pesticide analysis

Total moisture content in crude propolis was 3.5% w/w. About 113 pesticides were tested including phorate, ediphenphos, dimethoate and tricyclazole. All pesticides were found to be absent in EEIP.

3.5. In vitro cell line study

In vitro anti-cancer activity of EEIP was investigated and compared with the pure CAPE against human breast cancer MCF-7 and colon cancer HT-29 cells using *in vitro* SRB assay. The result illustrated in Table 2 indicates that EEIP showed better activity than pure CAPE. The total growth inhibition (TGI) concentration value of EEIP was found to be (31.10 μg/mL) and (39.90 μg/mL) whereas CAPE was 46.00 μg/mL and 47.20 μg/mL on MCF-7 breast cancer cells and HT-29 colon cancer cell lines respectively. Fig. 3(A–F) is the representative microscopic images obtained from *in vitro* anti-cancer studies on colon cancer cell line HT-29 and breast cancer cell line MCF-7. Results and microscopic images (Fig. 3(A–F)) showed that in Fig. 3(A&D) cells appeared more dense which are of normal control group of HT-9 and MCF-7 respectively. CAPE treated group on HT-9 and MCF-7 (Fig. 3B&E) showed less dense and rounded cells. Similarly more reduction in density and compact rounded cells were observed in EEIP treated group (Fig. 3C&F) on HT-29 and MCF-7 cells respectively.

Table 2
TGI and GI_{50} values of ADR, CAPE and EEIP on HT-29 colon cancer cell line and MCF-7 breast cancer cell line.

Samples	HT-29 cell line TGI (μg/mL)	HT-29 cell line GI_{50} (μg/mL)	MCF-7 cell line TGI (μg/mL)	MCF-7 cell line GI_{50} (μg/mL)
ADR	< 10	< 10	< 10	< 10
CAPE	47.20 ± 0.10	20.10 ± 0.050	46.00 ± 0.020	12.1 ± 0.010
EEIP	39.90 ± 0.020	16.50 ± 0.010	31.10 ± 0.015	< 10

Values are presented as mean ($n = 3$).
EEIP – ethanolic extract of Indian Propolis; CAPE – caffeic acid phenethyl ester; ADR – adriamycin positive control; TGI – concentration of drug that produce total inhibition of cells; GI50 – concentration of drug that produce 50% inhibition of cells; MCF 7 – human breast cancer cell line; HT-29 – human colon cancer cell line; μg/mL – microgram per milliliter.

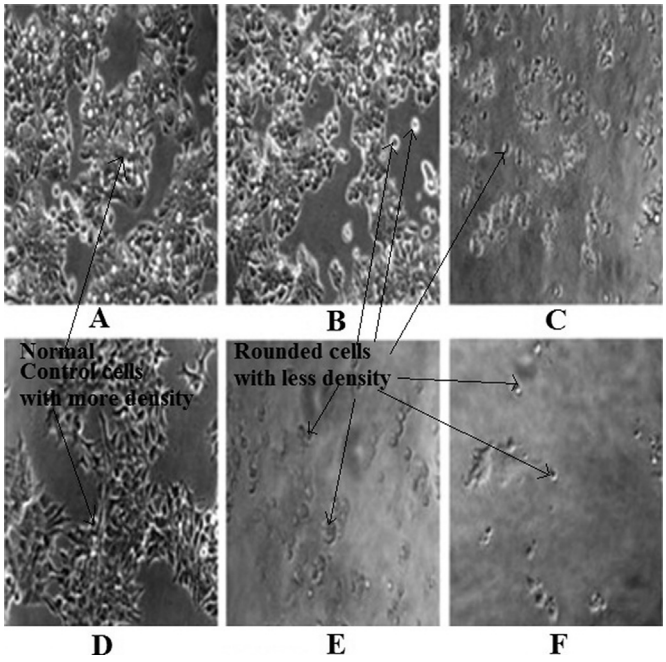

Fig. 3. *In vitro* cytotoxicity study on HT-29 cell line: A) Normal control cells (HT-29), B) CAPE treated, C) EEIP treated and *in vitro* cytotoxicity study on MCF-7 cell lines, D) normal control cells (MCF-7), E) CAPE treated and F) EEIP treated.

3.6. Hemolysis study and plasma protein binding

The hemolytic potential of the EEIP was evaluated using optical density method. The result indicates that the hemolysis rates for 0.25, 0.5, 0.75, 1.0 and 1.5 mg/mL concentration of EEIP were 1.5, 2, 2.8, 3.4 and 4.1% respectively.

The plasma protein binding rate for EEIP was obtained as 57.34 ± 1.36%.

3.7. In vivo anti-cancer study

Average life span of animal was found to be 48% in the DLA tumor control group, whereas average life span after 5-FU treatment was found to be 96%. EEIP at a dose of 100, 200, 400 mg/kg body weight showed percent increase in life span (Table 3) and these values were significant ($p < 0.01$). EEIP at a dose of 100, 200, 400 mg/kg body weight showed significant reduction ($p < 0.01$) in percent increase in body weight, packed cell volume and viable tumor cell count of animals when compared to DLA tumor bearing mice. As shown in Table 4, WBC count increased and Hb count, RBC count and platelets count decreased in the DLA control group (G2) as compared to normal control group (G1). Treatment with EEIP at a dose of 100, 200, 400 mg/kg body weight showed reversed changes in these values to about normal level.

The inoculation of DLA cells caused significant ($p < 0.01$) increase in the level of serum enzyme parameters in the tumor control group (G2) in comparison with the normal control group (G1). The treatment with EEIP at the dose of 100, 200 and 400 mg/kg body weight showed reversed changes in these values towards the normal level (Table 5). The treatment with standard 5-FU gave similar results.

4. Discussion

The new HPLC method was developed and validated for simultaneous estimation of selected markers. From the linear regression data it was found that the developed method is linear

Table 3
Effect of EEIP on the life span, body weight and cancer cell count of tumor induced mice.

Treatment groups	Number of animals	Percent increase in life span	Increase in body weight (g)	Cancer cell count (mL × 10^6)
G1	6	≫30 days	02.22 ± 0.68	–
G2	6	48%	9.44 ± 1.86[a]	2.75 ± 0.80[a]
G3	6	96%	5.66 ± 0.42[b]	1.30 ± 0.22[b]
G4	6	88%	5.45 ± 0.32[b]	1.42 ± 0.30[b]
G5	6	89%	5.36 ± 0.30[b]	1.40 ± 0.28[b]
G6	6	90%	5.30 ± 0.28[b]	1.38 ± 0.24[b]

G1 – normal control, G2 – cancer control, G3 – positive control, G4–G6– treatment control EEIP 100, 200, 400 mg/kg respectively.
All values are expressed as mean ± SD for 6 animals in each group.
One-way ANOVA followed by Newman–Keuls multiple comparison test.
[a] Values are significantly different from normal control (G1) at $p < 0.01$.
[b] Values are significantly different from cancer control (G2) at $p < 0.01$.

Table 4
Effect of EEIP on Hematological parameters.

Treatment groups	Total WBC (cells/ml × 10^3)	RBC count (millions/mm^3)	Hb (g/Dl)	Packed cell volume (%)	Platelets (Lakhs/mm^3)
G1	10.35 ± 1.05	4.55 ± 1.95	12.90 ± 1.95	14.25 ± 2.44	3.60 ± 0.95
G2	15.30 ± 2.60[a]	2.70 ± 0.98[a]	6.80 ± 0.95[a]	38.36 ± 3.35[a]	1.70 ± 0.42[a]
G3	12.30 ± 1.34[b]	4.05 ± 1.62[b]	11.90 ± 1.48[b]	16.40 ± 1.40[b]	2.94 ± 0.50[b]
G4	12.12 ± 1.26[b]	4.06 ± 1.50[b]	12.22 ± 1.52[b]	17.30 ± 2.36[b]	3.30 ± 0.65[b]
G5	12.05 ± 1.22[b]	4.08 ± 1.60[b]	12.25 ± 1.55[b]	17.24 ± 2.30[b]	3.36 ± 0.68[b]
G6	11.85 ± 1.18[b]	4.12 ± 1.65[b]	12.35 ± 1.60[b]	17.20 ± 2.26[b]	3.40 ± 0.70[b]

G1 – Normal control, G2 – Cancer control, G3 – Positive control, G4–G6 – Treatment control EEIP 100, 200, 400 mg/kg respectively.
All values are expressed as mean ± SD for 6 animals in each group.
One-way ANOVA followed by Newman–Keuls multiple comparison test.
[a] Values are significantly different from normal control (G1) at $p < 0.01$.
[b] Values are significantly different from cancer control (G2) at $p < 0.01$.

and sensitive. Baseline did not show any significant noise and there were no other interfering peaks around the retention time of caffeic acid, apigenin, quercetin and CAPE, indicating proposed RP-HPLC method is specific. The relative standard deviation values of the intra-day and interday precision study were within limit as per ICH guideline and method showed good precision. The proposed RP-HPLC method was found to be reliable for simultaneous quantification of selected markers and validation parameters are in the limits of ICH guidelines.

Results showed that the EEIP contains presence of apigenin, quercetin and CAPE whereas WEIP showed the presence of caffeic acid. Absence of markers was observed in hexane and ethyl acetate extracts.

In vitro anti-cancer activity of EEIP was investigated and compared with pure CAPE against human breast cancer MCF-7 and colon cancer HT-29 cells using *in vitro* Sulforhodamine B (SRB) assay. SRB assay is a well known and sensitive method for evaluating cytotoxic activity against both cancer and non-cancerous cell lines. It is advantageous over other contemporary cytotoxicity assays; it is independent of cell metabolic activity and also not interfered by test compounds. The GI 50 and TGI of EEIP were determined for two cancer cell lines by SRB assay. The cytotoxic ability of crude extracts can be attributed to their phytochemical constituents. The results obtained from GI 50, reveal that activity of both CAPE and EEIP was comparable to adriamycin and can be considered to have anti-cancer potential. The results showed that EEIP possesses comparatively better anti-cancer potential on MCF-7 breast cancer cell line than HT-29 human colon cancer cell line. Also, in both cell lines EEIP exhibited better anti-cancer potential than CAPE that may be because of synergistic activity of other polyphenols and flavonoids present in EEIP.

It has been postulated that polyphenols and flavonoids possess anti-cancer activity by several mechanisms including decrease of ROS, modulation of signaling pathways and down regulation of nuclear transcription factor kappa B (NF-κB). The reason for better growth inhibition on MCF-7 and HT-29 cell line might be due to synergistic effect of various polyphenols and flavonoids present in EEIP.

The acceptable hemolysis rate (less than 3%) [37] shown by EEIP denotes its non-hemolytic property up to 750 μg/ml. EEIP showed no or less effect on red blood cells. So the EEIP may be considered as biosafe for internal use. The plasma protein binding rate of EEIP was found in slightly higher range and indicates the need for development of suitable formulation to use EEIP internally as drug delivery system.

In *in vivo* anti-cancer activity, rapid increase in ascitic tumor volume was observed in DLA tumor bearing control group (G2). This ascitic fluid acts as a nutritional source for the growth of tumor cells [40]. Results showed that EEIP at a dose of 100, 200, 400 mg/kg body weight decreases the nutritional fluid volume, arrests the tumor growth and increases life span of DLA bearing mice which supports anti-tumor nature of EEIP. Myelosuppression and anemia are the common problems in cancer chemotherapy. Reduction in hemoglobin content results in anemia in tumor bearing mice because of iron deficiency, hemolysis or myelopathic conditions [41]. After treatment with EEIP at the dose of 100, 200 and 400 mg/kg, hemoglobin (Hb) content, RBC count, WBC count came to normal levels significantly. It indicates the protective action of EEIP at the dose of 100, 200 and 400 mg/kg on the hemopoietic system. The significantly elevated level of total cholesterol, TG, AST, ALT, ALP in serum of tumor inoculated animals indicated liver damage. EEIP at the dose of 100, 200 and 400 mg/kg significantly changed their levels to normal. Overall data supports the anti-tumor nature of EEIP. EEIP showed better *in vitro* and *in vivo* cytotoxicity potential on MCF-7 and HT-29 cell line as compared to CAPE which may be attributed to synergistic effects of various polyphenols and flavonoids in its composition.

Table 5
Effect of EEIP on serum enzymes and lipid proteins.

Treatment groups	Cholesterol (mg/dl)	TGL (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G1	108.85 ± 3.05	136.85 ± 2.55	36.40 ± 1.65	31.28 ± 1.45	132.28 ± 2.08
G2	146.95 ± 4.34[a]	220.28 ± 4.40[a]	78.6 ± 2.94[a]	62.32 ± 2.60[a]	265.30 ± 4.35[a]
G3	126.30 ± 3.84[b]	169.15 ± 2.65[b]	44.40 ± 1.72[b]	34.52 ± 1.70[b]	154.45 ± 2.40[b]
G4	117.26 ± 3.42[b]	160.08 ± 2.55[b]	42.44 ± 2.30[b]	35.28 ± 1.55[b]	162.45 ± 2.22[b]
G5	115.18 ± 3.38[b]	156.25 ± 2.50[b]	41.60 ± 2.20[b]	34.90 ± 1.42[b]	160.48 ± 2.18[b]
G6	113.36 ± 3.26[b]	153.30 ± 2.46[b]	40.90 ± 2.16[b]	34.80 ± 1.38[b]	158.45 ± 2.15[b]

G1 – Normal control, G2 – Cancer control, G3 – Positive control, G4–G6 – Treatment control EEIP 100, 200, 400 mg/kg respectively, Total Cholesterol (TC), Triglycerides (TGL), Aspartate amino Transferase (AST), Alanine amino Transferase (ALT), Alkaline phosphatase (ALP), U/L – units per liter.
All values are expressed as mean ± SD for 6 animals in each group.
One-way ANOVA followed by Newman–Keuls multiple comparison test.
[a] Values are significantly different from normal control (G1) at $p < 0.01$.
[b] Values are significantly different from cancer control (G2) at $p < 0.01$.

5. Conclusion

New, simple, precise and reliable HPLC method for simultaneous estimation of caffeic acid, apigenin, quercetin and CAPE was developed and different extracts of Indian propolis have been standardized. EEIP was selected on basis of standardization and chemical analysis. The polyphenols and flavonoid rich EEIP exhibited better *in vitro* anti-cancer activity than pure CAPE, a potent anti-cancer constituent of propolis. Antitumor activity *in vivo* reveals that EEIP was effective in inhibiting the tumor progression, most likely because of synergistic activity of constituents present in the extract. However, the exact molecular mechanism by which EEIP mediates its anti-tumor activity is to be studied. From pesticidal analysis, hemolysis and plasma protein binding studies it can be concluded that the EEIP is safe for internal use and can be considered for development of suitable formulation. Based on the above promising results, further development of suitable formulation for CAPE and EEIP and its *in vivo* antitumor study are in process.

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Conflict of interest

None

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