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Formulation and Evaluation of Combinational Liposomes of Biochanin-A and Docetaxel for Breast Cancer Treatment

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***Abstract*- The solubility study of Biochanin A (BCA) and Docetaxel (DTX) indicated that both drugs were poorly soluble in water but shown the better solubility in HBSE buffer. So, the HBSE buffer was chosen as vehicle since sufficient amount of drug dissolve in it, which is necessary to maintain sink conditions. The partition coefficient of BCA and DTX was found to be 3.42 and 4.38 respectively. The values clearly indicated that both the drugs are lipophilic and soluble in lipids and so both the drugs have been added to the lipid part during preparation of liposome. Various formulations of the combination of BCA and DTX were prepared by sonication method with different ratios of soya lecithin and cholesterol at different sonication speed. Entrapment efficiency of the F6 formulation of Biochanin A was very high (70.0±0.58) then F6 formulation of Docetaxel (69.9±0.96) because of high sonication speed. The effect of the soya lecithin and cholesterol ratio in the lipid components of vesicles on the entrapment efficiency of lipophilic drugs, Biochanin A and Docetaxel, the efficiency was increased with sonication speed and decreased with the ratio of phosphatidylcholine. Particle size of liposomes was measured by particle size analyzer and best size of liposome formulation vehicle was 202±0.66 nm of formulations F6. MTT assay for selected formulations were performed and it was found that combination liposomes were havingbetter cytotoxic potential than the individual drugs (separate drugs / drugs alone). The difference was statistically significant.**

***Keywords:* Biochanin-A, cancer, cholesterol, cytotoxic potential, docetaxel, lipophilic, liposomes, nano-particles, phosphatidylcholine, sonication, soya lecithin.**

INTRODUCTION

Cancer

Cancer is a broad term to encompass several malignant diseases. In cancer, cells divide and grow uncontrollably, forming malignant tumors andinvade in nearby organs / body parts of the body. Fitzmaurice *et al*., 2017, the World Health Organization (WHO) illustrated that cancer is second leading cause of mortality / death all over the globe (globally 1in 6 deaths is due to cancer). There are more than 200 different types of cancer.

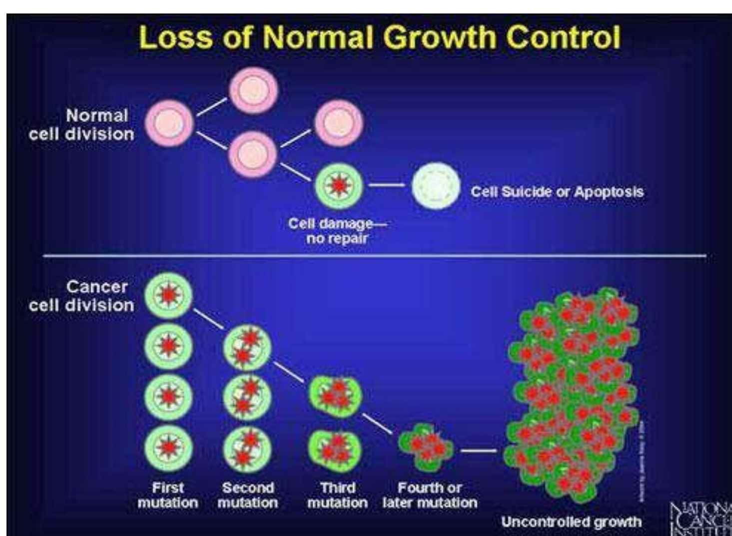


Figure 1: Cancer's Schematic illustration (Terry, 2008).

In cancer, cells divide and grow uncontrollably, forming malignant tumors and invade in nearby organs / body parts of the body. A tumor can be benign or malignant as follows:

Benign Tumor	:	This is not cancer and cells are confined to one area and are not able to spread to other parts of the body.
Malignant Tumor	:	Made up of cancerous cells, which have the ability to spread by travelling through the bloodstream or lymph fluid.

Pathogenesis of Cancer (Wang, 2008)

Step	Details
Initiation	First step in the two-stage model of cancer development. The effects of initiators are irreversible;
Promotion	There are two general categories of promoters: specific promoters that interact with receptors on or in target cells of defined tissues and nonspecific promoters that alter gene expression without the presence of a known receptor.
Progression	Progression refers to the stepwise transformation of a benign tumor to a neoplasm and to malignancy. (Figure 2)

Treatment of Cancer

Most people have a combination of treatments, such as surgery with chemotherapy and/or radiation therapy (patients have several cycles of treatment over a number of weeks or months) as follows (Table 1)

Table 1: Combination of treatments available for Cancer (Meena, 2020)

Surgery	:	Procedure in which a surgeon removes remove cancerous tissue and some healthy tissue around it (may be a major, invasive operation or a relatively minor procedure).
Radiation Therapy	:	Uses radiation, such as x-rays or gamma rays (high doses), to kill cancer cells or shrink tumors or injure them so they cannot multiply; External beam radiotherapy or internal radiotherapy.
Chemotherapy	:	Cytotoxics drugs kill or slow the growth of cancer cells.
Targeted Therapy	:	Targeted therapy attacks specific genetic changes (mutations), while minimising harm to healthy cells.
Hormone (endocrine) therapy	:	Hormone therapy uses synthetic hormones to block the effect of the body's natural hormones that help some cancers to grow (breast and prostate cancers).
Immunotherapy to Treat Cancer	:	Drugs are used to stimulate the body's immune system to recognise and fight some types of cancer cells.
Stem Cell Transplant	:	Procedure that restored blood-forming stem cells in cancer (in patients subjected to high doses of chemotherapy or radiation).
Precision Medicine	:	Helps doctors select treatments that are most likely to help patients based on a genetic understanding of their disease.

Breast Cancer and Treatment Choices

Breast cancer is the most commonly diagnosed form of solid tumors and is the second leading cause of death in western women. About 20-30% women with breast cancers develop metastatic breast cancers. Breast cancer research has increased dramatically during the last two decades, resulting in an extraordinary progress in our understanding of the disease, and in more efficient, and less toxic treatment options (Jang *et al*., 2003).

Types of Breast Cancer

- Ductal: Cancer forms in the milk ducts
- Lobular: Cancer originates in milk producing lobules.
- Ductal Carcinoma in Situ (DCIS)
- Invasive (or Infiltrating) ductal carcinoma (IDC)
- Invasive (or Infiltrating) lobular carcinoma (ILC)
- Inflammatory breast cancer (IBC)
- Triple Negative Breast Cancer

- Lobular carcinoma in situ (LCIS)
- Paget's disease of the nipple

Etiology and Risk Factors of Breast Cancer (Wang, 2008)

Lopez *et al*., 2010, etiology of several breast cancers is unknown, however several risk factors appear to increase the probability of breast cancer. These include:

- Female Gender and Age
- Personal history of cancer
- Family history of cancer and genetics
- Hormonal Factors
- Benign Breast Disease
- Obesity and Dietary Fat
- Radiation exposure

How breast cancer spreads

Breast cancer can spread when the cancer cells get into the blood or lymph system and then are carried to other parts of the body. The lymph vessels carry lymph fluid away from the breast. In the case of breast cancer, cancer cells can enter those lymph vessels and start to grow in lymph nodes.

Treatment of Breast Cancer

The treatment choices for breast cancer can be categorized into local or systemic therapy. Surgery, radiation therapy, hyperthermia, and photodynamic therapy are examples of localized therapy. Surgery is the treatment of choice for breast cancer which involves lumpectomy, partial mastectomy or quadrantectomy, and mastectomy.

Radiation therapy is an adjuvant therapy in which high energy radiation is used to kill cancer cells remaining in the breast, chest wall, underarm area after the surgery. Local therapy is beneficial for non-metastatic forms of breast cancers whereas systemic therapy is required for metastatic forms of breast cancers. Systemic therapy by means of chemotherapy, hormonal therapy, immunotherapy or targeted therapy, and gene therapy involves administration of the respective therapeutic agent by the oral or parenteral route.

Chemotherapy

Perez & Fernández, 2015, Chemotherapy is the use of drugs to kill cancer cells. It can be used as adjuvant therapy after surgery for patients with no evidence of metastasis. Adjuvant chemotherapy reduces the risk of recurrence of cancer. Chemotherapy can also be used as a neoadjuvant therapy to surgery wherein the drugs are administered before surgery to shrink the tumor so that it can be removed easily by surgery. For chemotherapy to be effective, the drug should be delivered at the tumor sites at adequate concentrations. Inadequate concentrations of the drug at the tumor microenvironment could result in regrowth of the tumor cells and development of resistance (Jain, 1998).

Liposomes in Drug Delivery

Sharma (1997), Nanoparticles have been studied for the past two decades in drug delivery with numerous materials as drug delivery vesicles, such as mesoporous silica, carbon nanotubes, graphene sheets, liposomes, micelles, dendrimers, polymer-drug self-assemblies, *etc*. Various controllable drug delivery systems have been developed with either the initiative properties of the vesicles or proper modifications with other molecules or nanoparticles (Sawant *et al*., 2010; Xia *et al*., 2014).

Liposome, approved to be safe in clinic practices by FDA, has been regarded as one of the most promising and practical approaches to controllable drug delivery in medical applications, especially in chemotherapy for cancer treatment. Due to the biocompatibility, biodegradability, low toxicity and immunogenicity, liposomes have attracted intensive attention in the past decades in the field of drug delivery (Figure 2).

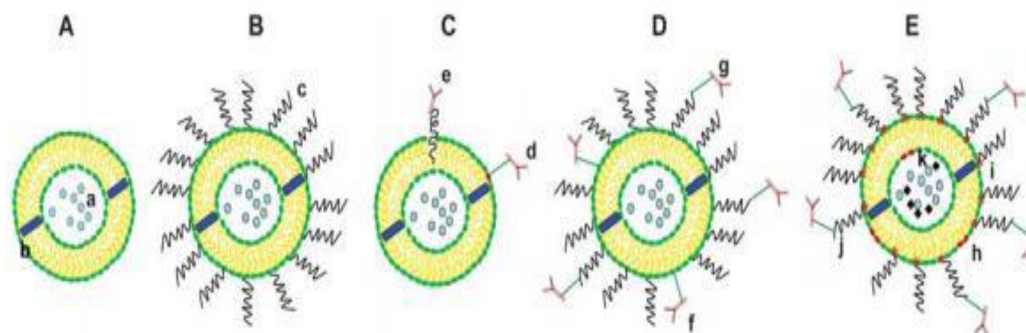


Figure 2. A. Early stage plain liposomes with water soluble/insoluble drugs; B. Long circulating liposomes grafted with PEG or other protective polymers; C. Targeted liposomes modified with antibodies; D. Long circulating liposomes with protective polymers and targeting antibodies; E. Long circulating liposomes loaded with stimuli responsive cargos leading to more controllable system with protective polymers and targeting antibodies.

Liposomes: An ideal "Drug Carrier" for anticancer drugs

Sayed (1989), anticancer drugs (are known to produce serious side effects to other healthy tissues. The more serious effects are myocardiopathy and pulmonary toxicity. Therefore targeting such type drugs to the cancerous cell is essential because these drugs are new for the treatment of different type of carcinomas effectively. Selective delivery of the entrapped material to specific tissues with minimal losses of drug during transit, regulation of the drug delivery rate, reduction of toxicity and removal of unused drug. All these function can co-exist in a single liposome preparation which makes it an ideal carrier of drug. Liposomes have proved to be suitable vehicles for antitumor drugs. For example, doxorubicin which attacks dividing cells rapidly is used in the treatment of malignant tumors. Doxorubicin's most serious side effect is progressive and irreversible damage to the heart. In addition the drug attacks hair follicles, intestinal cells and cells of immune system suppression.

Liposomes for Breast Cancer

Chonn & Cullis, 1995, Breast cancer is the most common type of cancer in females, constituting the second leading cause of death globally. The treatment of breast cancer is complex and relies on several factors, including the type of tumor, tumor size, grade, proliferation rate and lymph node status.

Docetaxel as a Chemotherapeutic Agent

Wani *et al*., 1971, the first taxane, paclitaxel, was identified as the cytotoxic compound from the crude extract of the North American pacific yew tree. Gelmon, (1994), however, the scarce supply of the pacific yew tree and the difficulties of paclitaxel formulation led to the discovery of a taxane derivative, docetaxel, which is produced from 10-deacethylbaccatin-III found in the European yew tree. Docetaxel is a molecule with an anhydrous molecular weight of 807.9 and a chemical formula of $C_{43}H_{53}NO_{14}$ (Figure 3).

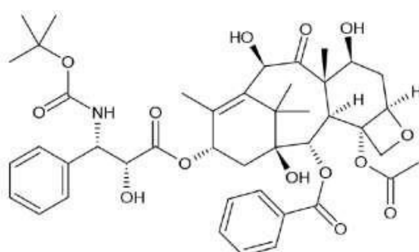


Figure 3: The Structure of Docetaxel.

Lecithin-in-Water Emulsions Nanoparticles

Cui *et al*., 2006, The nanoparticles used in my research are composed of lecithin and polysorbate 20 (Tween 20) and prepared via the emulsion precursor. This technique provides several advantages. It avoids the use of organic solvent, the high-torque mechanical mixing or the homogenization. Jimenez *et al*., 1990, illustrated that lecithin is a complex mixture of phosphatidesconsisting of phosphatidylcholine, phosphatidylserine,phosphatidylinositol and other substances such as triglycerides and fatty acids. Present research works was been undertaken with following objectives:

- Formulation of combinational liposomes of DTX and BCA.
- Characterization of combinational liposomes of DTX and BCA.
- Evaluation of combinational liposomes of DTX and BCA.
- Assessment of liposomal stability of combinational liposomes.

Materials and Methods

Drug Profile: Docetaxel (DTX)

Wani *et al*., 1971, DTX is produced from 10-deacethylbaccatin-III found in the yew tree. Docetaxel is a molecule with an anhydrous molecular weight of 807.9 and a chemical formula of $C_{43}H_{53}NO_{14}$ (Rao *et al*., 2006).

Drawbacks of Docetaxel Therapy

Engels *et al*., 2007, adverse reactions due to either the drug itself (neurotoxicity and musculotoxicity) or solvent system (hypersensitivity & fluid retention) have been reported (Persohn *et al*., 2005).

Biochanin A (Bio A / BCA)

Sartorelli, (2009), summarised that Biochanin A (BCA) is present in *Trifolium medium, Trifolium pretense*, *Trifolium incarnatum*, *Trifolium arvense*, *Trifolium pannonicum* and *Trifolium rubens* its concentration is lower (Breikaa, 2013). Lee *et al*., 1991, BCA block the vasoconstriction in a dose-dependent manner dueto the inhibition of L-type calcium channels.

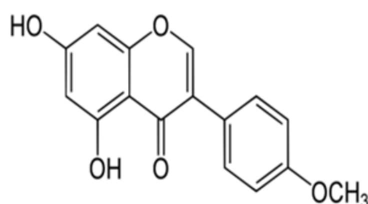


Figure 4: Chemical Structure of Biochanin A (BioA / BCA).

Pre-formulations Studies

A. Identification of Drugs DTX and BCA

- Identification of Drug by FTIR
- Identification of Drug by Ultraviolet (UV) Spectroscopy
- Melting Point (mp) Determination
- Physical Appearance Analysis
- Partition Coefficient Determination

B. Purity Determination of Drug (DTX and BCA)

- Determination of Drug by Standard Curve
- Purity Determination of Drug by Solubility Analysis

Identification of Drug (DTX and BCA) by FTIR

- The KBr (spectroscopic grade) pellets were prepared and examined by FTIR (8400S, Shimadzu Corporation, Japan);
- 1 mg of Docetaxel was mixed with 10 mg of KBr (spectroscopic grade) in a glass mortar.
- The scanning was done using KBr dispersion pellets (moisture free);
- Scanned between 4000-400 cm^{-1};
- Characteristic FTIR peaks attributable to functional groups present in the drug molecule to establish its identity and purity.

Identification of Drug DTX and BCA by Ultraviolet (UV) Spectroscopy

In order to determine λmax of DTX and BCA stock solution were prepared and analysed for absorbance at wavelength between 400-550 nm using Double Beam UV Spectrophotometer - Model 2202, Systronics. λmax were observed.

Melting Point Analysis of DTX and BCA

Melting point (mp) is the temperature at which the pure liquid and solid exist equilibrium. In practice, it is taken

as equilibrium mixture at an external pressure of 1 atmosphere, this is sometimes known as normal melting point. The thiels tube method of melting point determination in liquid paraffin was used in the present investigation.

Physical Appearance Analysis of DTX

Colour	:	White Powder
Nature	:	Crystalline Powder (Solid)
Odour	:	Odorless
Taste	:	Tasteless

Physical Appearance Analysis of BCA

Colour	:	White to Off –White Powder
Nature	:	Anhydrus Powder (Solid)
Odour	:	Characteristic
Taste	:	Characteristic

Partition Coefficient Determination of DTX and BCA

- PC is ratio of unionized drug distributed between the organic and aq. Phase;
- For a drug delivery system, lipophilic/hydrophilic balance has shown to be a contributing factor for the rate and extent of drug absorption;
- PC characterize and determines lipophilic/hydrophilic nature of drug.
- Measurement of drug lipophilicity indicated its ability to cross the lipoidal cell membrane oil/water partition coefficient system such as octanol/water, octanol / 0.1NHCl etc. The octanol / water partition coefficient (Kow) is defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two phase octanol / water system.

Kow = concentration in octanol phase / concentration in aqueous phase

Preparation of Standard Curve

Preparation of Stock Solution

1 ml solution (drug : ethanol :: 1:1 w/v) was taken and mixed with 10 ml ethanol.

Preparation of Aliquots (Dilutions)

10 different dilutions were prepared like 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 µg/ml, 80 µg/ml, 90 µg/ml and 100 µg/ml from the stock solution. Absorbance was recorded by using UV spectrophotometer (Model 2202, Systronic, India).

Solubility Studies of DTX and BCA

The solubility studies were performed in HBSE buffer solution (pH 7.4) and 10% HBSE buffer by adding excess amount of drug in each case and keeping the excess drug containing HBSE buffer on a water bath shaker for 24 hrs at 32^0C. (Aroa *et al*., 2002).

Drug-excipients Interactions Studies by FTIR (Malik *et al*., 2011)

- 1 part of drug sample and 5 parts of potassium bromide (KBr) was taken;
- Mixed thoroughly in a mortar while grinding with the pestle;
- Mixed powder was pressed at 5000-10000 psi to form a translucent pellet;
- Pellet placed in FTIR sample holder through which the beam can pass.
- Drug DTX was combined with BCA and pellets were prepared and analysed;

Formulation Development

Preparation of Liposomes (Averineni *et al*., 2009)

- Liposomes, small unilamellar vesicles (SUVs) were prepared by the sonication method;
- Nine formulations were prepared;
- In the first three formulations (F1-F3), ratio of soya lecithin and cholesterol were ranged varied as 9:1,8:2, 7:3 respectively and sonicated at 25000 rpm;
- In the same way, Formulations F4-F6 were prepared but sonication (30,000 rpm);
- Formulation F7 and F10 were taken as blank for MTT assay;

- ❖ Phosphatidylcholine and cholesterol were weighed and dissolved in chloroform (3-4 ml). BCA and DTX were weighed and dissolved;
- ❖ Flask was attached to rotary evaporator at 45.0^0C in water bath under vacuum;
- ❖ Liquid evaporated and a dry thin lipid film was deposited on the walls of the flask;
- ❖ Flask were left in a vacuum desiccators overnight to ensure removal of solvents;
- ❖ The appropriate volume of HBSE buffer (10 mM HEPES, 150mM NaCl, 9.1 mM EDTA, pH 7.5) (preheated at 60^0C) was added and the vessel vigorously agitated on a rotary mixer to produce multilamellar vesicles (MLVs);
- ❖ The MLVs were then sonicated at 60^0C for 15 min. to produce unilamellar liposome;
- ❖ After sonication, the liposome samples were incubated at 60^0C for 15 minutes to allowed them to anneal;

Table 2: Composition of Liposomal Formulations.

Formula-tions	DTX + BCA (mg)	Phosphati-dylcholine	Cholesterol	Sonication Speed (rpm)
F1	500 + 100	9	1	25,000
F2	500 + 100	8	2	25,000
F3	500 + 100	7	3	25,000
F4	500 + 100	9	1	30,000
F5	500 + 100	8	2	30,000
F6	500 + 100	7	3	30,000
F7	--	9	1	25,000
F8	500	8	2	25,000
F9	100	7	3	25,000
F10	--	9	1	30,000
F11	500	8	2	30,000
F12	100	7	3	30,000

Evaluation of Formulations

Drug Entrapment Efficiency (Averineni *et al*., 2009)

Drug entrapment efficiency was determined by the mini-column centrifugation method. The concentration of the encapsulated drug was estimated by the UV spectrophotometric method (Patel *et al*., 2009). Each determination was made in triplicate. The percentage of drug entrapment and yield was calculated according to the following equation:

$$\text{Percentage (\%) Entrapment} = \frac{\text{Total Drug} - \text{Diffused Drug} \times 100}{\text{Total Drug}}$$

Morphological Studies

Particle Size Measurement / Particle Size Analysis

The particle size of the lipososmes was measured using a particle size analyser (Malvern Master Sizer 2000, UK). The liposomes size distribution profile was determined by light scattering based on the laser diffraction method. Liposomes were immersed in oil medium before measurement at 25 ± 1^0C by scattering light at 90^0C.

In-vitro Cytotoxicity Studies of Liposome (MTT Assay)

Cytotoxicity in cell lines (Mossman *et al*., 1983)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is taken up by the viable cells and reduced to formazan by the "Succinate – tetrazolium reductase" system in the mitochondrial respiratory chain of metabolically active cells. Formazan formed, is a purple colored water-insoluble product that is largely impermeable to cell membranes, thus resulting in its accumulation within the healthy cells which is solubilised by adding DMSO. The optical density (OD) of purple colored solution developed was read using a conventional ELISA plate reader at 540 nm. The % cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{(\text{Abs of Control} - \text{Abs of blank}) - (\text{Abs of Test-Abs of Blank})}{(\text{Abs of Control} - \text{Abs of blank})} \text{X}100$$

Viability rate = (mean of control-mean of treatment) / (mean of control) × 100

Results

Identification of DTX and BCA

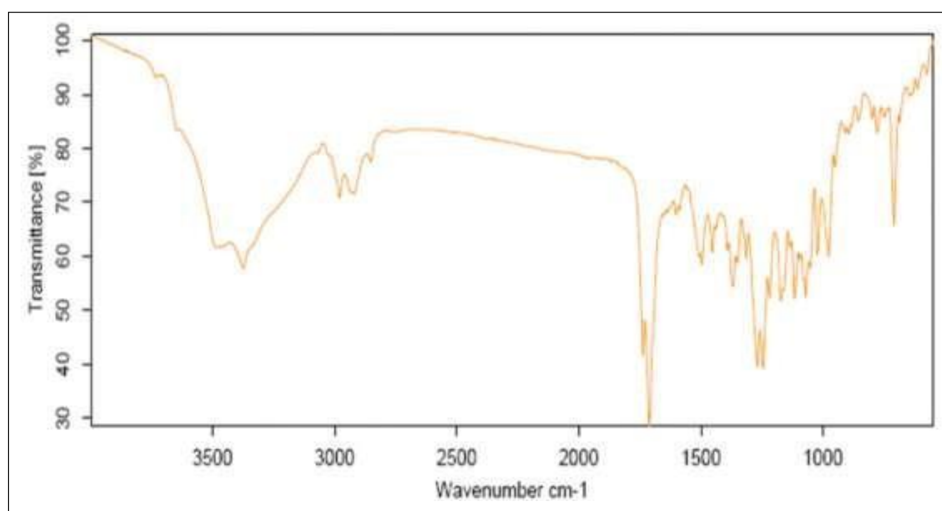
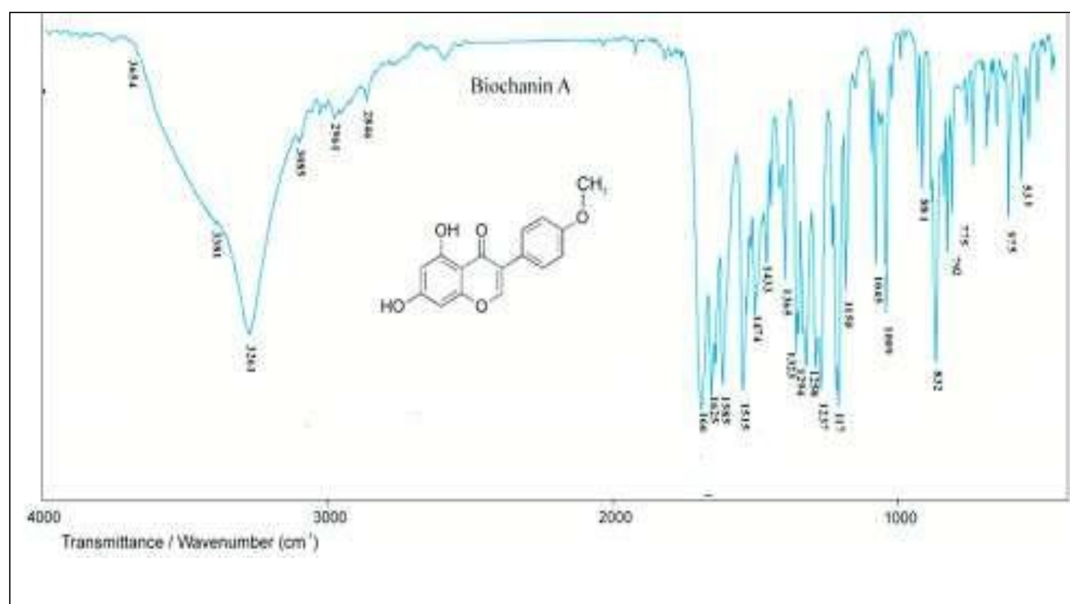


Figure 5 : FTIR Spectrum of Docetaxel.

Interpretation of FTIR spectrum of Docetaxel showed (Figure 5) peaks (cm^{-1}) at 3316 (N-H; Stretching; secondary amine), 2973 (N-H Stretching; secondary amine), 2885 (C-H Stretching; alkane), 1636 (N-H Bending), 1447 (C-H Bending; vibration of CH_2 and CH_3), 1359 (O-H Bending; carboxylic group), 1104 (C-O Bending; carbonyl group), 1057 (C-N Bending; amide group) and (C=C Bending; alkane group).



Interpretation of FTIR spectrum of BCA (Figure 6) showed peaks (cm^{-1}) at 3261 (O-H Bending), 1176 (C-O Bending), 1323 (O-H Stretching), 1661 (C=O Bending), 1625 (C=C Bending), 1585 (C=C Bending), 1515 (C=C Bending), 1258 (C-O-C Bond), 1237 (C-O-C Bond).

Identification of DTX and BCA by Ultraviolet (UV) Spectroscopy

The UV-visible spectrum obtained by scanning the 10µg/ml of DTX recorded between 200 nm to 400 nm. It was found that DTX showed λmax at 202 nm (Figure 7).Further, it was found that BCA showed λmax at 261 nm (Figure 8).

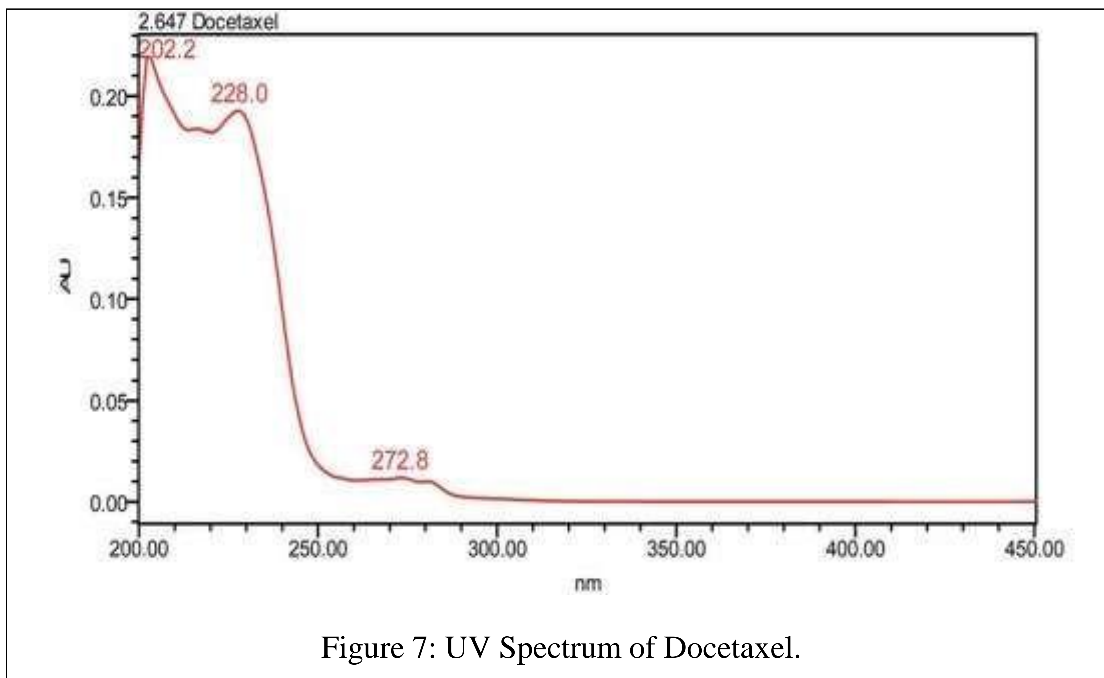


Figure 7: UV Spectrum of Docetaxel.

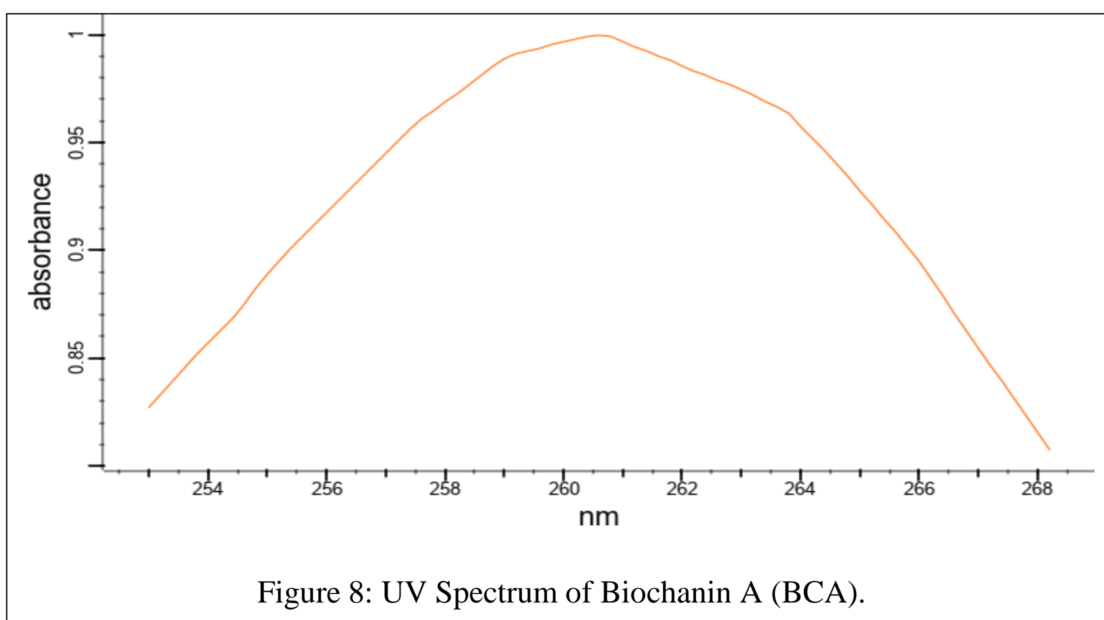


Figure 8: UV Spectrum of Biochanin A (BCA).

Melting Point Analysis

Thiels-tube method was used.

Table 3: Melting Point of Docetaxel (DTX) and Biochanin A (BCA).

Drug	Reported Melting Point	Observed Melting Point
Docetaxel (DTX)	232^0C	232^0C
Biochanin A (BCA)	210^0C-213^0C	211.5^0C

Physical Appearance Analysis

Docetaxel (DTX) was found to white to off-white amorphous powder (solid) having no odour and no taste. Besides, it was found that DTX was freely soluble in DMSO, tetra-hydrofuran, Ethanol, acetone, Methanol, Chloroform, ethyl acetate. Biochanin A was found to be white crystalline powder which poorly soluble in water whereas soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF), and acetone.

Partition Coefficient Determination

Table 4: Partition Coefficient of Docetaxel.

Concentration of DTX			
	Mean Absorbance	Mean Concentration (µg/ml)	Partition Coefficient
Octanol	0.095	79	4.38
Water	0.020	18	

Table 5: Partition Coefficient of Biochanin A.

Concentration of Biochanin A			
	Mean Absorbance	Mean Concentration (µg/ml)	Partition Coefficient
Octanol	2.340	72	3.42
Water	0.711	21	

The partition coefficient was determined using the formula and the values of BCA and DTX were found to be 3.42 and 4.38 respectively which means both the drugs were lipophilic in nature.

Preparation of Standard Curve

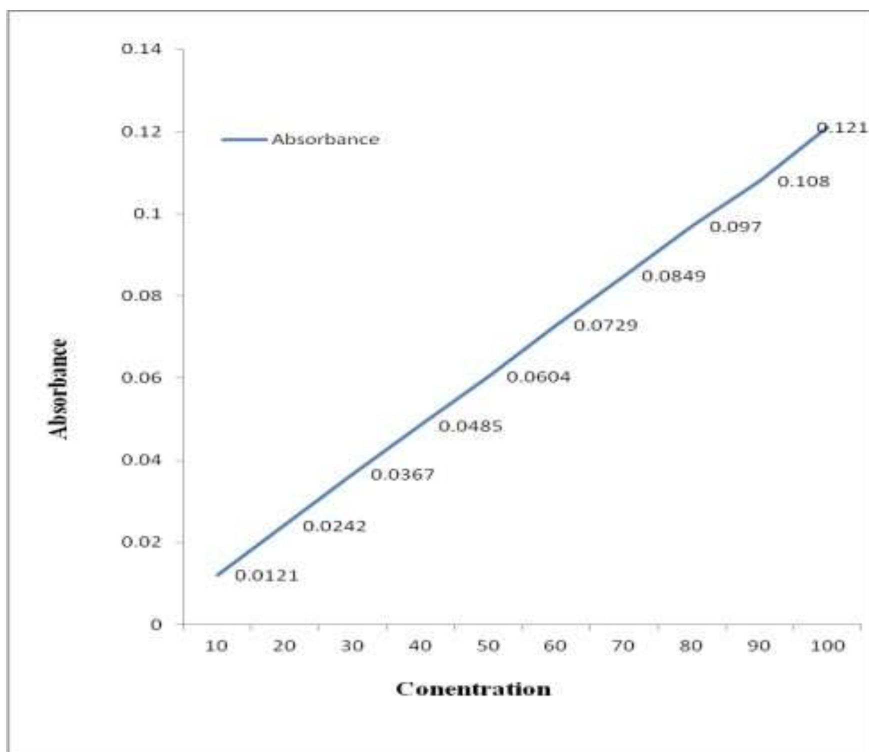


Figure 9: Calibration Curve of DTX in Ethanol at 275 nm.

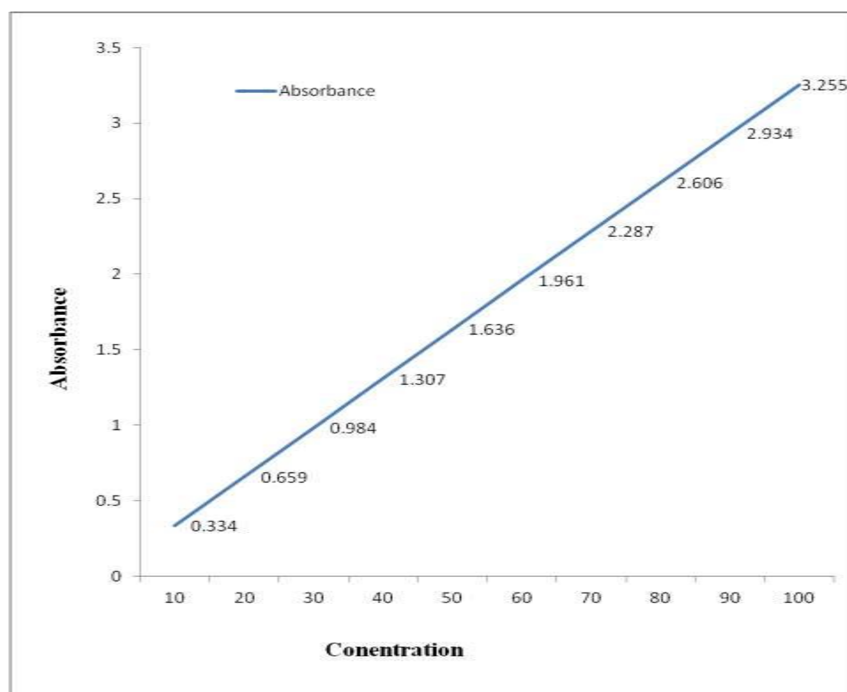


Figure 10: Calibration Curve of BCA.

Solubility Studies

Table 6: Solubility of Docetaxel (DTX).

S. No.	Solvent	Mean Solubility ± SD (µg/10ml)	Mean Solubility ± SD (µg/ml)
1.	Water	38.15 ± 13.5	3.815 ± 1.35
2.	Phosphate buffer saline, pH 7.4	46.82 ± 4.2	4.682 ± 0.42
3.	HBSE buffer	127.8 ± 8.6	12.78 ± 0.86

Table 7: Solubility of Biochanin A (BCA).

S. No.	Solvent	Mean Solubility ± SD (µg/10ml)	Mean Solubility ± SD (µg/ml)
1.	Water	1307 ± 165.0	130.7 ± 16.50
2.	Phosphate buffer saline, pH 7.4	3105 ± 118.9	310.5 ± 11.89
3.	HBSE buffer	9000 ± 95.6	900.0 ± 9.56

It was found that drug was less soluble in posphate buffer saline than HBSE buffer. The HBSE buffer was chosen because sufficient amount of drug dissolve in it, which is necessary to maintain sink conditions.

Drug-excipients Interactions Studies by FTIR

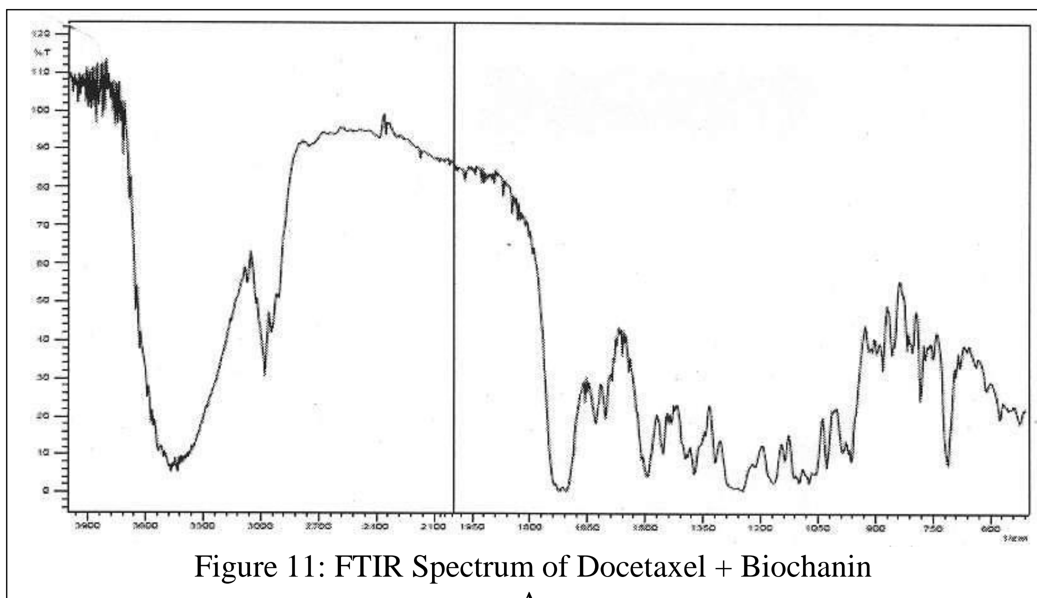


Figure 11: FTIR Spectrum of Docetaxel + Biochanin A

Drug-excipient interaction study was carried out by FTIR to ensure that there was no interaction between them. Insignificant changes were observed which clearly indicated that there was no physical / chemical interaction between the drug- excipients.

Evaluation of Formulations
Drug Entrapment Efficiency

Table 8: Drug Entrapment Efficiency of DTX and BCA.

Formula-tions	Conc.of DTX (µg/ml)		% Entrapment of DTX ±SEM	Conc.of BCA (µg/ml)		% Entrapment of BCA ±SEM
	In Supernatant	In Pellet		In Supernatant	In Pellet	
F1	48.3	36.0	51.7 ± 4.05	189.2	310.1	62.16 ± 3.6
F2	46.8	37.9	53.2 ± 3.68	180.0	318.5	64.0 ± 1.08
F3	41.0	40.3	59.0 ± 2.7	169.1	320.2	66.18 ± 4.12
F4	44.7	39.5	55.3 ± 2.68	175.7	316.2	64.86 ± 1.86
F5	33.5	45.2	66.5 ± 1.62	153.3	341.8	69.34 ± 2.06
F6	30.1	50.0	69.9 ± 0.96	150.0	349.7	70.0 ± 0.58
F7	0.0	0.0	0.0	0.0	0.0	0.0
F8	0.0	0.0	0.0	199.5	300.0	60.1 ± 4.15
F9	49.8	35.1	50.24 ± 4.78	0.0	0.0	0.0
F10	0.0	0.0	0.0	0.0	0.0	0.0
F11	0.0	0.0	0.0	196.9	305.1	60.62 ± 3.97
F12	49.2	35.5	50.8 ± 3.8	0.0	0.0	0.0

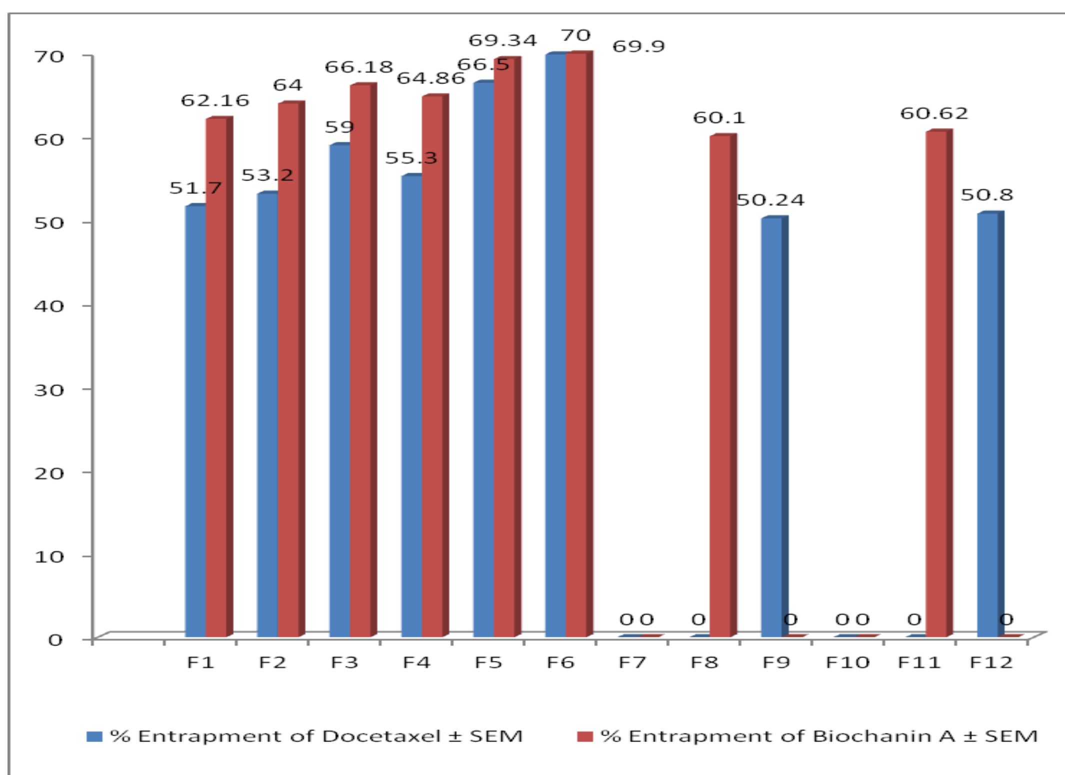


Figure 12: Entrapment of Docetaxel (DTX) and Biochanin A (BCA).

Particle Size Measurement

The particle size of formulations was determined by Zeta Sizer (Malvern) and the mean particle size (in nm) of all formulations with standard error of mean (SEM) was recorded and presented in Figure 13.

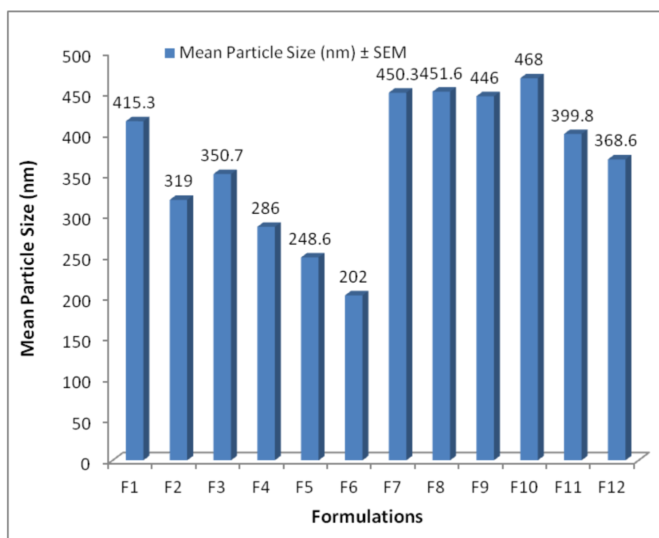


Figure 13: Particle Size analysis of Formulations.

***In-vitro* Cytotoxicity Studies of Liposomes (MTT Assay)**

Table 9: MTT Assay.

Formulations	Read at 540 nm			Average	CV	% Cell Death
F12	0.33	0.269	0.322	0.307	10.8	25.9
F11	0.255	0.248	0.243	0.249	2.4	40.0
F6	0.143	0.132	0.18	0.152	16.6	63.4
F10	0.0286	0.0264	0.036	0.0303	1.6	3.06

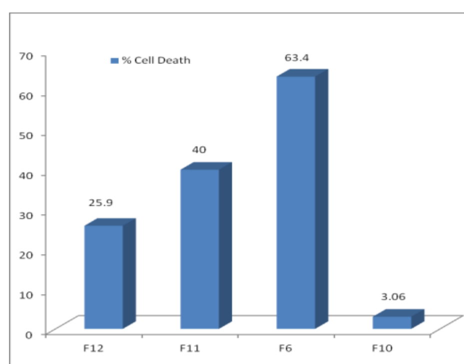


Figure 14: MTT Assay.

Conclusion

In present research, on the basis of experimental results it has been concluded that BCA and DTX loaded liposomes were successfully developed. The ratios of phosphatidylcholine and cholesterol and sonication speed in the formulations were varied to optimize them. Various conclusions were drawn as BCA and DTX loaded liposomes with phosphatidylcholine andcholesterol can be easily and scientifically prepared very rapidly, best optimized ratio of soya lecithin and cholesterol was (7:3), change in sonication speed reflects the change in the particle size of the formulation, order of particle size among the formulations were F6>F5>F3>F4>F2> F12>F1>F11>F7>F8>F9>F10, encapsulation efficiency of BCA among the formulation were in order F6>F5>F3>F4>F2> F1>F11>F8 and the encapsulation efficiency of DTX among the formulation were in order F6>F5> F3>F4>F2>F1>F12>F9. Thus, finally it has been concluded that soya lecithin and cholesterol can be used as avehicle for developing liposome in ratio of 7:3 for delivery of combination drugs like BCA and DTX. Cytoxicity activity of developed liposome was tested against the hormone dependent breast cancer cell line (MCF-7). Further, it is proposed that liposome should be tested against non-hormone dependent breast cancer and other cancer cell lines. The *in-vitro* and *in-vivo* activities will certainly prove its utility as a chemotherapeutic agent.

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