UNIVERSITI PUTRA MALAYSIA

EFFECT OF ZERUMBONE-LOADED NANOSTRUCTURED LIPID CARRIER ON CANINE MAMMARY GLAND TUMOUR CELL LINE

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EFFECT OF ZERUMBONE-LOADED NANOSTRUCTURED LIPID CARRIER ON CANINE MAMMARY GLAND TUMOUR CELL LINE.

FOONG JIA NING

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Effect Of Zerumbone-Loaded Nanostructured Lipid Carrier On Canine Mammary Gland Tumour Cell Line", by Foong Jia Ning and in our opinion is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
AOPI	Acridine Orange and Propodium Iodide
CMT	Canine mammary gland tumour
DOX	Doxorubicin
DMSO	Dimethylsulphoxide
FBS	Foetal Bovine Serum
FPV	Faculty of Veterinary Medicine
GI50	Growth Inhibition Dose 50
hr	Hour
IBS	Institute Bioscience
IU	International Unit
LD50	Lethal Dose 50
µL	Micro-litre
mL	Millilitre
M	Molar
NLC	Nanostructure Lipid Carrier
PBS	Phosphate Buffer Solution
RPMI	Roswell Park Memorial Institute
SLN	Solid Lipid Nanoparticles
SPSS	Statistical Package for the Social Sciences
UPM	University Putra Malaysia
UVH	University Veterinary Hospital
ZER	Zerumbone
ZER-NLC	Zerumbone-loaded Nanostructure Lipid Carrier

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan VPD 4999-Projek.

KESAN PEMBAWA LIPID NANOSTRUKTUR TERISI ZERUMBON TERHADAP TITISAN SEL TUMOR KELENJAR MAMA KANIN

Oleh

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2015

Penyelia: Prof. Dr Rasedee Abdullah

Zerumbon (ZER) daripada rizom halia liar, *Zingiber zerumbet* (L.) Smith adalah suatu sebatian diet semula jadi lipofilik yang diketahui mempunyai sifat antitumor, antikeradangan, antioksidan, antimikrob, antinosiseptif, pelindung hati dan imunopemodulatan. Bagaimanapun, penggunaan terapi ZER diganggui oleh kelarutan air buruk yang mengakibatkan kepada penyerapan and biokeperolehan and penghantaran kepada tisu sasar rendah. Untuk mengatasi kelemahan ini, ZER telah diisikan ke dalam pembawa lipid nanostuktur (NLC) (ZER-NLC). Dalam kajian ini kesan antikanser ZER-NLC ditentukan terhadap titisan sel (CMT-stylo) kelenjar mama kanin. Adalah dipostulat bahawa pengisian ZER ke dalam NLC tidak menjejas sifat

antikanser ZER. Menguna assai MTT (3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromida), daya hidup titisan sel CMT-stylo selepas perlakuan dengan 1.7 mM of ZER adalah 64.30±9.87, 42.06±9.00, 37.81±10.04% dan dengan 1.8 mM ZER-NLC adalah 102.77±12.68, 38.42±9.16, 41.13±11.72%, masing-masing pada 24, 48 and 72 jam perlakuan. Dos maut separuh maksimum (LD50) untuk ZER dan ZER-NLC selepas 72 perlakuan, masing-masing ialah 100 dan 90 µM. Dos perencatan pertumbuhan separuh maksimum (GI50) untuk ZER dan ZER-NLC selepas 72 jam perlakuan, masing-masing adalah 20 dan 25 µm. Kesan antikanser ZER dan ZER-NLC juga digambarkan mengguna kaedah pewarnaan dedua akridina jingga/propidium iodida. Zerumbon dan ZER-NLC mengaruh apoptosis terhadap sel CMT-stylo yang ternyata sebagai pembleban membran, penjidaran nukleus dan pengkondensasian kromatin. Kajian ini kali pertamanya menunjukkan yang ZER-NLC sebagai sistem penghantar drug ada potensi berkesan dalam rawatan tumor kelenjar mama kanin. ZER-NLC adalah inovatif, novel, selamat untuk terapi kanser.

Kata kunci: zerumbon, pembawa lipid nanostruktur, pembawa lipid nanostruktur terisi zerumbon, tumor kelenjar mama kanin, antikanser, apoptosis.

ABSTRACT

An abstract of the project paper presented to Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999-Project.

EFFECT OF ZERUMBONE-LOADED NANOSTRUCTURED LIPID CARRIER ON CANINE MAMMARY GLAND TUMOUR CELL LINE.

By

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2015

Supervisor: Prof. Dr. Rasedee Abdullah

Zerumbone (ZER) from the rhizomes of the wild ginger, *Zingiber zerumbet* (L.) Smith, is a natural dietary lipophilic compound with antitumour, antiinflammatory, antioxidant, antimicrobial, antinociceptive, hepatoprotective and immunomodulatory properties. However, therapeutic application of zerumbone is plagued by poor water-solubility and subsequent poor absorption, bioavailability and delivery to target tissues. To overcome this limitation, ZER was loaded into nanostructured lipid carrier (NLC) (ZER-NLC). In this study the anticancer effect of ZER-NLC was determined on a canine mammary gland tumour (CMT-stylo) cell line. It is postulated that loading of ZER into NLC does not compromise the anticancer properties ZER. Using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, the proliferation of CMT-stylo cells after treament with 1.7 mM of ZER was at 64.30±9.87, 42.06±9.00,

37.81±10.04% while with 1.8 mM ZER-NLC at 102.77±12.68, 38.42±9.16, 41.13±11.72% after 24, 48 and 72 hr treatment, respectively. The half maximal lethal dose (LD50) for ZER and ZER-NLC at 72 hr was 100 and 90 µM, respectively. The half maximal growth inhibition dose (GI50) for ZER and ZER-NLC after 72 hr treatment was 20 and 25 µM, respectively. The anticancer effect of ZER and ZER-NLC was also visualised using the acridine orange/propidium iodide double staining method. Zerumbone and ZER-NLC induced apoptosis of CMT-stylo cells as shown by the membrane blebbing, nucleus margination and chromatin condensation. This study, for the first time, shows that ZER-NLC is a potentially effective drug delivery system for the treatment of canine mammary gland tumours. The ZER-NLC is innovative, novel, and safe, for cancer therapy.

Keywords: zerumbone, nanostructured lipid carrier, zerumbone-loaded nanostructured lipid carrier, canine mammary gland tumour, anticancer, apoptosis.

1.0 INTRODUCTION

Zerumbone (ZER), is a major compound from the essential volatile oil of edible wild ginger rhizomes, *Zingiber zerumbet* (L.) Smith (Kitayama *et al.,* 2003; Rahman *et al.,* 2013). It has been shown to possess antitumor, antiinflammatory, antioxidant, antimicrobial, antinociceptive, hepatoprotective and immunomodulatory activity (Abdelwahab *et al.,* 2009; 2011; Rahman *et al.,* 2013). However, therapeutic application of zerumbone has been plagued by poor water solubility and subsequent poor absorption, bioavailability and delivery to target tissues and organs eventhough it has been credited with numerous pharmacological potentials (Rahman *et al.,* 2013; Rahman *et al.,* 2014). Therefore, alternative method of drug administration; for example, a drug carrier system is needed to overcome this problem. Beginning in 1990s, advances in nanotechnology has resulted in the production of a lipid carrier, nanostructured lipid carriers (NLC) that is able to load lipophilic drug to enhance drug delivery, loading stability, controlled-release and tolerability of drugs (Abbasalipourkabir *et al.,* 2011b).

In order to overcome ZER poor water-solubility and subsequently increase its therapeutic application, ZER can be loaded into NLC, which is an effective drug carrier for the compound (Abdelwahab *et al.,* 2011; Abbasalipourkabir *et al.,* 2011b; Souto, 2004; Müller *et al.,* 2000). A study

of apoptogenic effect of ZER-loaded NLC (ZER-NLC) on an acute human lymphoblastic leukemia (Jurkat) cell line *in vitro* showed that ZER-NLC is a potentially effective drug delivery system for the treatment of leukemia (Rahman *et al*., 2014). To date, there is no study done on the anticancer effect of ZER-NLC on canine mammary gland tumour cell line.

Therefore, the objective of this project is to determine the anticancer effect of ZER-NLC on a canine mammary gland tumour cell line (CMT-stylo). We hypothesised that ZER-NLC has cytotoxic effect on the CMT-stylo cell line via the induction of apoptosis and the incorporation of ZER into NLC does not alter the anticancer effect of ZER.

The objectives of this study are to:

1) determine the anticancer effect of ZER-NLC on the mammary gland tumour (CMT-stylo) cell line.
2) compare the cytotoxic effects of ZER and ZER-NLC.
3) determine the mechanism of cytotoxicity of ZER-NLC on CMT-stylo cell line.

2.0 LITERATURE REVIEW

2.1 Canine Mammary Gland Tumour

Canine mammary gland tumour (CMT) is a neoplasia of the epithelial or myoepithelial tissues of the mammary glands (Sorenmo, 2003; Gough and Thomas, 2004). It is the most common tumour in the intact female dog and the second most common tumour in all dogs (Sorenmo, 2003; Sleeckx *et al*., 2014). Approximately 50% of the tumours are benign, of which 50% are malignant. Approximately 75% of canine mammary gland tumour can be cured by surgery alone, which means that the remaining 25% of patients are at high risk of metastasis (Cronin, 2010).

Approximately 40% of all CMTs are located in the inguinal mammary glands and appear shortly after oestrus. The incidence of tumour is higher at the posterior pairs of mammary gland (caudal abdominal and inguinal pairs of mammary gland) and this correlates with the larger gland and abundant secretion during lactation (Baba and Câtoi, 2007).

Canine mammary gland tumour, especially malignant tumour, is rare in dogs less than 2 years of age, but the risk increases with age especially by 6 to 10 years old. The peak incidence age is dependent on breed of dogs. In general, larger breeds have a naturally shorter lifespan and therefore tend to be younger than smaller breeds when they are diagnosed with mammary

gland tumour. The average age of dogs at diagnosis is 10-11 years (Baba and Câtoi, 2007).

The incidence of CMT is higher in certain breeds, for example the: - toy and miniature Poodle, German Shepherd, English Spaniel, Brittany Spaniel, English Setter, Pointer, Fox Terrier, Boston Terrier and Cocker Spaniel. On the contrary, its incidence is lower in the Chihuahua and Boxer breeds (Bostock, 1986).

There is correlation between obesity and the development of this tumour. According to a study done by Lim *et al.*, (2015), the mean age of mammary gland tumour development was lower in overweight or obese dogs (9.0 ± 1.8 years) than in lean dogs or optimal bodyweight (10.2 ± 2.9 years), and the evidence of lymphatic invasion of carcinoma cells was found more frequently in overweight or obese than in lean or optimal dogs.

Canine mammary gland tumour is influenced by the effect of hormones particularly estrogen and progesterone. Thus, CMT can be preventable by neutering female dogs. Spaying of dogs via ovariohysterectomy that removes the source of estrogen and progesterone before they come into first estrus would decrease the risk of developing mammary tumours to approximately 0.5% in their lifetime (O'Keefe, 1995). The protective effect

of ovariohysterectomy decreases tremendously by the first few estrous cycles. It has been shown that, ovariohysterectomy is no longer beneficial once the dog reaches 4 years of age (Kristiansen *et al*., 2013). The incidence of CMT after the first estrous cycle is approximately 8% and after the second estrous cycle, more than 26% (Baba and Câtoi, 2007).

2.2 Zerumbone

Zerumbone, a crystalline, monocyclic, sesquiterpene, phytochemical, lipophilic compound with low water solubility (1.296 mg/L at 25°C) can be extracted from the essential volatile oil of edible wild ginger rhizomes, *Zingiber zerumbet* (L.) Smith (Kitayama *et al.,* 2003; Rahman *et al.*, 2013). It has been shown to possess antitumour, anti-inflammatory, antioxidant, antimicrobial, anti-nociceptive, hepatoprotective and immunomodulatory activity (Abdelwahab *et al.,* 2009; 2011; Rahman *et al.,* 2013). This compound has been shown to have antiproliferative effects on human colon cancer (HT-29, CaCo-2), liver cancer (HepG2), (Chien *et al.*, 2008), human leukemia (Jurkat) (Rahman *et al.,* 2013) and cervical cancer (HeLa) cell lines (Abdelwahab *et al.,* 2009). Zerumbone has shown *in vivo* to have chemopreventive properties on skin cancer and cervical intraepithelial neoplasia in mice (Murakami *et al*., 2004), and colon cancer in rats (Tanaka *et al*., 2001). Cytotoxic effect of ZER is selective toward cancer cells and relatively harmless to normal Chang liver cells (Sharifah Sakinah *et al*.,

2007), human dermal (2F0-C25), colon (CCD-18 Co) fibroblasts (Murakami *et al*., 2002) and peripheral blood mononuclear cells (Rahman *et al*., 2014).

2.3 Nanostructured Lipid Carrier

Nanoscience that began to explode in the 1990s has brought new chapters to the treatment of diseases. Solid lipid nanoparticles (SLN) were developed early at the beginning of 1990s as an alternative carrier system to the existing traditional carriers, such as emulsions, liposomes and polymeric nanoparticles which are plague by low solubility and bioavailability, adverse effect, suboptimal therapeutic index, expensive production method (Müller *et al.*, 2000; Cho *et al.*, 2008). Solid lipid nanoparticles was a promising alternative carrier system due to its exceptional stability, low toxicity, enhanced absortion rate, scaling-up potential and biocompatible components (Radomska-Soukharev, 2007; Hu *et al*., 2004). However, SLN as a drug carrier poses several disadvantages such as drug leakage during storage, tendency for particle growth, unpredictable gelation tendency, unexpected dynamics of polymorphic transitions and inherently low incorporation capacities due to the crystalline structure of solid lipids (Westesen *et al*., 1997; Westesen and Siekmann, 1997; Souto, 2004; Wang *et al.*, 2012). The inability of the SLN to retain the loaded drugs is attributed

to the highly-ordered crystalline lipid matrix that facilitates uncontrolled release of drugs from the nanoparticle (Rahman *et al.,* 2014).

To overcome the limitations of SLN, NLC was developed (Müller *et al*., 2000). Nanostructured lipid carrier is the second generation SLN, which is composed of solid lipid matrix incorporated into liquid lipids, producing crystalline imperfections that allow for greater drug entrapment (How *et al*., 2013). Since liquid lipids are better solubilisers of drugs than solid lipids, incorporation of drugs into NLCs increase drug-loading while minimising drug expulsion (Guimarães and Maria, 2011).

Nanostructured lipid carriers are very small, thus they are minimally phagocytosed by macrophages of the mononuclear phagocytic system (Mühlen *et al*., 1998) and not destroyed in the body (How *et al.,* 2011). Moreover, its small size and superior particle surface to volume ratio increase loading efficiency and bioavailability of drug, making it a promising drug-delivery system **(**Abbasalipourkabir *et al.,* 2011b). The NLCs, because of its small size, also come in close contact with the stratum corneum, resulting in increased drug penetration through the mucosa or skin (Souto, 2004).

Among the advantages of NLC are possibility of encapsulation of lipophilic drugs without the toxic effect of the carrier, high rate of drug entrapment efficiency (EE) that can result in long-term physical stability of the drug, flexibility in route of administration (can be either oral, parenteral, ocular, dermal, or pulmonary) (Chinsriwongkul *et al*., 2011; Weber *et al*., 2014; Üner *et al.*, 2014) and a potential of large scale production (Souto, 2004; How *et al.*, 2011; 2013).

2.4 Zerumbone-loaded Nanostructured Lipid Carrier

Zerumbone can be loaded into NLC by the hot, high-pressure homogenization technique to produce a ZER-NLC. Incorporation of ZER into NLC protects the compound from chemical degradation while facilitating controlled release (Abbasalipourkabir *et al*., 2011a). The ZER-NLC particles have an average size of 52.68 ± 0.1 nm, a polydispersity index of 0.29 ± 0.004 μm and zeta potential of - 25.03 ± 1.24 mV. The particles were relatively uniform and spherical in shape (Rahman *et al.*, 2013)

Due to the high lipophilicity of ZER, the entrapment efficiency of NLC was 99.03%, and the drug-loading capacity was 7.92%, which is the result of high solubility in the lipid matrix.

The *in vitro* drug release of ZER from ZER-NLC was 46.7%, following a zero-order equation, with pure zerumbone dispersion of 90.5% over 48 hours (Rahman *et al*., 2013). This showed that released of compounds from NLC is a slow, controlled release manner, making ZER-NLC an effective system for prolonged cancer therapy (Rahman *et al*., 2014).

On the human T-cell acute lymphoblastic leukemia (Jurkat) cells, ZER-NLC has a half maximal inhibitory concentration (IC50) of 5.64 ± 0.38 µg/mL, while for free ZER it was 5.39 ± 0.43 µg/mL after 72 hours of treatment. The ZER-NLC induce apoptosis of leukemia cells via the intrinsic mitochondrial pathway of apoptosis (Rahman *et al*., 2013; 2014).

2.5 Doxorubicin Hydrochloride

Current available treatment option for CMT is mainly surgical excision and/or chemotherapy using cytotoxic drug like vincristine or doxorubicin. However, current cancer chemotherapy can damage or kill the rapid dividing and healthy cells of the host and also causes adverse effects such as nausea, anaemia, and hair loss. Furthermore, the cost of chemotherapy using current drugs is higher (Alabsi *et al*., 2012).

Doxorubicin, also known as Adriamycin, is a cytotoxic anthracycline antibiotic isolated from the cultures of *Streptomyces peucetius* var. *caesius*.

It is a reddish-orange lyophilized photosensitive powder that is freely soluble in water. It is one of the most widely used antineoplastic drugs in the human and veterinary medicine (Hill *et al.*, 2014). Doxorubicin is most cytotoxic to cardiac cells, followed by melanoma, sarcoma cells, and normal muscle, skin fibroblasts and rapidly proliferating normal cells such as bone marrow, hair follicles, and, gastrointestinal tract mucosa (Plumb, 2008).

Doxorubicin must be administered intravenously as it is not absorbed through the gastrointestinal tract. It is extremely irritating to tissues if administered subcutaneously or intramuscularly (Plumb, 2008). Thus, personal protective equipment must be worn when preparing and administrating the drug. Doxorubicin also causes several adverse effects including bone marrow suppression, cardiac toxicity, alopecia, gastroenteritis, and stomatitis. In dogs, doxorubicin causes immediate hypersensitivity reaction that is characterized by urticaria, facial swelling, vomiting, arrhythmias, and/or hypotension.

3.0 MATERIALS AND METHODS

3.1 Canine Mammary Tumour Cell Line

A canine mammary tumour (CMT-stylo) cell line (passage 11) was used in this study, courtesy of Dr. Teo Guan Young and Dr. How Chee Wun of Institute of Bioscience, UPM and Professor Dr. Rasedee Abdullah of the Faculty of Veterinary Medicine, UPM. This primary cell line was obtained from an excised mammary gland adenocarcinoma of a 13-year-old local female canine patient treated at the University Veterinary Hospital, UPM.

The cells were maintained in Iscove's Modified Dulbecco's medium (IMDM; GibcoBRL) supplemented with 10% foetal bovine serum (FBS; HyClone), 100 units/mL penicillin and 100 µg/mL streptomycin (GibcoBRL) at 37^{o}C in a humidified atmosphere with 5% CO_2, and 95% air (Figure 1).

3.2 Zerumbone, Zerumbone-Loaded Nanostructured Lipid Carrier, Nanostructured Lipid Carrier

Preparation of Zerumbone

Pure colorless ZER crystals were prepared from the extracted essential oil of fresh *Z. Zerumbet* rhizomes by steam hydrodistillation. Initially, the fresh

rhizomes were cleaned, washed, sliced and then placed in a steam distillator containing tap water and heated. The distillate containing volatile oil was collected in a Dienstag glassware. The volatile oil was then crystallised using absolute n-hexane 100% (Sigma-Aldrich, USA) and the solution was then left to evaporate in a fume hood (Novaire, Newton, MA, USA). Recrystallization was performed three times, also using absolute n-hexane (100%) to obtain pure zerumbone crystals (Rahman *et al*., 2013) (Figure 1).

Preparation of Nanostructured Lipid Carrier

The NLC were prepared by high-pressure homogenization technique (How *et al.,* 2011). The lipid phase, composed of hydrogenated palm oil, olive oil, and lipoid S100 at a ratio of 7:3:3, was melted by heating to approximately 10^{o}C above the melting point of the lipid matrices to avoid lipid memory effect (Figure 1).

Preparation of Zerumbone-loaded Nanostructured Lipid Carrier

Zerumbone (0.4%, w/v) in lipid melt was dispersed in the aqueous surfactant solution to obtain hot pre-emulsions by high-speed stirring in an Ultra Turrax® (IKA Werke GmbH and Co, Staufen, Germany) at 13,000 rpm for 10 minutes. The hot pre-emulsions were then homogenized in a high-pressure homogenizer (EmulsiFlex®, Avestin Inc, Ottawa, ON, Canada) at 1,000 bar for 20 cycles at 60°C. The hot oil-in-water

nanoemulsion was immediately transferred to siliconised glass vials, sealed, and allowed to undergo lipid phase crystallization at room temperature (25^{o}C) for formation of ZER-NLC (Figure 1) (Rahman *et al.*, 2013).

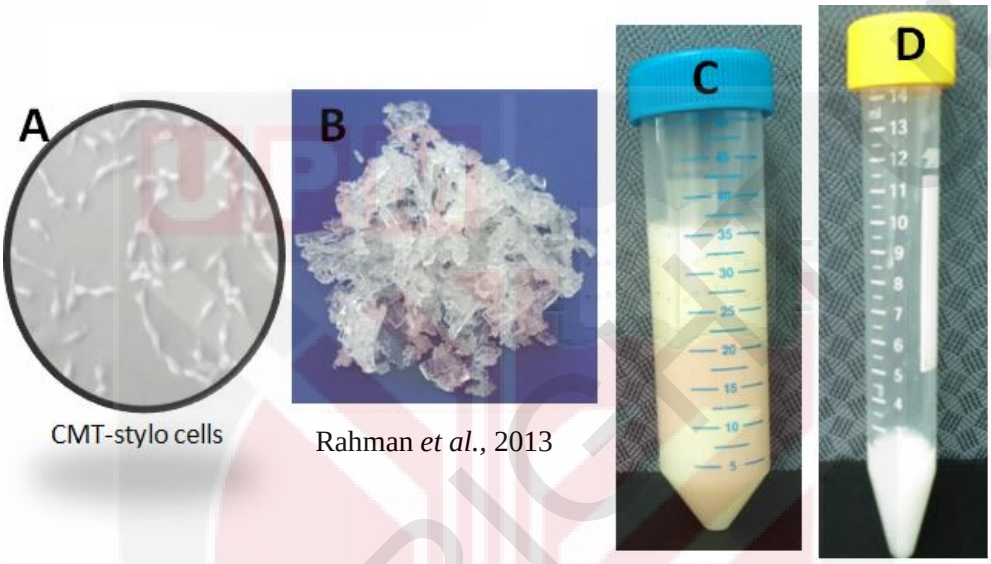

Figure 1: (A) Canine mammary gland tumour (CMT-stylo) cells, (B) pure Zerumbone crystal, (C) Nanostructured Lipid Carrier suspension (D) Zerumbone-loaded Nanostructured Lipid Carrier suspension.

3.3 Cell Seeding and Treatment

Cell seeding

Cells grown to near confluence in T-75 cm^2 flasks were trypsinised (0.05% trypsin, 0.02% EDTA in phosphate buffered saline (PBS) and counted using a hemocytometer. One hundred microliter suspension containing 0.5 x 10^4 CMT-stylo cells in growth medium were then seeded into 60 wells of the 96 well flat-base microculture plates. The outer periphery rows only contain

growth media. The cells were then incubated for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Treatments with Zerumbone, Nanostructured Lipid Carrier and Zerumbone-loaded Nanostructured Lipid Carrier

The ZER stock and working solutions were prepared using dimethyl sulfoxide solution (Sigma-Aldrich) and RPMI-1640 complete growth medium (Invitrogen Corp., Auckland, New Zealand), respectively, whilst the ZER-free NLC and ZER-NLC working solutions were prepared with RPMI-1640 complete growing medium. A cell suspension containing approximately 5 x 10^4 cells/mL was then seeded into each well of 96-well microculture plates (Techno Plastic Products AG, Trasadingen, Switzerland). Simultaneously, the cells were treated with 100 µL of 6.5, 12.5, 25, 50 and 100 µM of either Doxorubicin (positive control), ZER, blank NLC, and ZER-NLC various concentrations in the 96-well flat-base microculture plates (Appendix 1). Negative control wells received 100 µL of medium. The plates were then incubated for 24, 48 and 72 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

3.3.1 Cell Proliferative Assay

The plates were subjected to MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine cell viability. Twenty microliter of MTT stock solution (5 mg/mL, Sigma-Aldrich®, USA) in pH 7.4 phosphate-buffered solution (Sigma-Aldrich Co. LLC., USA) was added to each well. The plates were then incubated in the dark for another 3 hr for viable cells to convert MTT to a water insoluble purple formazan dye. The media were aspirated and remaining purple formazan was dissolved in 150 µL of Dimethylsulphoxide (DMSO; Fisher Scientific, USA) 5 min shaking. The plates were then incubated for another 30 min to completely dissolve the dye. The concentration of the purple dye is directly proportional to the number of viable cells which is detected spectrophotometrically using an enzyme-linked immunosorbent assay universal plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA) at 570 nm after background correction at 630 nm. The half maximal growth inhibitory concentration (GI50), median lethal dose (LD50) and cytostatic concentration values were determined from the dose-response curves. Results were expressed as the percentage of cell proliferation with respect to the negative control. Finally, the antiproliferative effect of ZER-NLC was compared with that of ZER, NLC and doxorubicin.

3.3.2 Cells seeding

The cells grown to near confluence in T-75 cm^2 flasks were trypsinised and counted using a hemocytometer. One milliliter suspension containing $5x10^4$ CMT-stylo cells in growth medium were then seeded into each well a 6-well flat-based plate (Becton Dickinson Labware, Franklin Lakes, NJ USA). Two plates were seeded (Appendix 2) and the cells then incubated for 24 hr in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Treatments

The cells were treated with 1000 µL of ZER, NLC, and ZER-NLC at their LD50, Cytostatic dose and GI50 concentrations in the 6-well flat-base plates respectively. The plates were then incubated for 24, 48 and 72 hr in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Doxorubicin was used as positive control, while negative control well was treated with media only.

3.3.3 Cell Morphology

3.3.3.1 Inverted light Microscopy

The cells were then viewed under an inverted microscope and the morphology of the cells captured at 24, 48 and 72 hr of incubation.

3.3.3.2 Acridine Orange (AO) and Propodium Iodide (PI) Staining

After 72 hr of incubation, the cells were trypsinised and harvested and centrifuged for 5 min at 900 rpm (Centrifuge 5810 R, Eppendorf AG, Germany) and 4^{o}C. The supernatant was discarded and the cells washed twice with PBS by centrifuging at 900 rpm (Centrifuge 5810 R, Eppendorf AG, Germany) for 5 min. Ten microliter of fluorescence dyes containing acridine orange (AO, 10 mg/mL) (Invitrogen^{TM}, USA) and propidium iodide (PI, 10 mg/mL) (Sigma-Aldrich Co. LLC., USA) were added to the cell pellets. Ten microliter of freshly stained cell suspension was dropped onto a glass slide, covered by coverslip, and viewed under UV-fluorescence microscope (Zeiss Axio Vert. A1, USA) within 30 min before the fluorescence colour starts to fade.

3.4 Statistical Analysis

The results expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and *Post hoc* Tukey test were performed on the data using Statistical Package for the Social Sciences version 20.0 software (SPSS Inc, Chicago, IL, USA). Probability value of $\alpha<0.05$ were considered to be statistically significant.

4.0 RESULTS AND DISCUSSION

4.1 Cell Proliferation

The proliferation of CMT-stylo cells treated with ZER was 64.3±9.87, 42.06±9.00, 37.81±10.04%; while, for ZER-NLC, it was 102.77±12.68, 38.42±9.16, 41.13±11.72% after 24, 48 and 72 hr of treatment, respectively. The proliferation of CMT-stylo cells treated with ZER was significantly (p<0.05) higher than those treated with ZER-NLC after 24 hr. However, no significant (p>0.05) difference was observed between treatments after 48 and 72 hr. Zerumbone treatment produced significantly (p<0.05) lower proliferation of CMT-stylo cells than NLC treatment after 48 and 72 hr (Figure 2).

The GI50 value for ZER on CMT-stylo cells was 40.5, 18.75 and 20 µM; while for ZER-NLC; it was 67.25, 17.75 and 25 µM after 24, 48, and 72 hr of treatment, respectively (Figure 5). The cytotoxic effect of ZER on CMT-stylo cells was similar to that of ZER-NLC after 48 and 72 hr of treatment. However, there was significant (p<0.05) difference in cell proliferation between ZER and ZER-NLC treatments after 24 hr.

The slightly higher proliferation of CMT-stylo cells treated with ZER-NLC than those treated with ZER after 24 hr may be due to the slow released of ZER from its lipid carrier. Pure ZER exerts its full effect almost

immediately upon exposure. This phenomenon typifies the controlled drug release characteristic of loaded NLC. Nanostructured lipid carrier with the incorporation of liquid and solid lipids produces a crystalline imperfection nanoparticle, which allows for high entrapment efficiency.

However, at 48 and 72 hr, the CMT-stylo cell proliferation did not differ significantly ($p>0.05$) between ZER and ZER-NLC treatments. This is because ZER in ZER-NLC had been released to cause similar cytotoxic effect to CMT-stylo cells (Rahman *et al.*, 2013). The only possible difference between ZER-NLC and pure ZER is in their mode of action, is that pure ZER is immediately available to target cells upon administration, whereas, for ZER from ZER-NLC to exert its effect, the nanoparticle must first be internalised through endocytosis or phagocytosis to release ZER into the extracellular space (Chen *et al*., 2008).

These characteristics of drug-loaded NLC, not only makes it an efficient drug carrier for ZER but facilitates long term physical stability of the loaded drug (Jithan and Swathi, 2010; Abbasalipourkabir *et al*., 2011a). Several mechanisms are involved in release of drugs from nanoparticles, to include surface and bulk erosion, disintegration, diffusion and desorption (Rahman *et al*., 2013).

The LD50 value for ZER was 100 µM; while for ZER-NLC, it was 90 µM after 72 hr of treatment (Figure 6). It is obvious that median lethal dose of ZER is similar to that of ZER-NLC. The cytostatic dose for ZER was 76, 61 and 45 µM and for ZER-NLC, it was 89.5 57.5 and 50 µM after 24, 48 and 72 hr of treatment, respectively (Figure 7). The result suggests that the cytostatic doses of ZER-NLC on CMT-stylo cells were also similar to ZER.

The proliferation of CMT-stylo cells treated with NLC was 69.38±5.64, 61.19±3.41 and 73.89±4.0% after 24, 48, 72 hr of treatment, respectively. The NLC causes some degree of cytotoxicity on CMT-stylo cells. The cytotoxic effect of NLC is due to its adherence to the cell membrane, internalization and degradation of cellular components. Although nanoparticles are formulated with glycerides consisting of fatty acids which are safe and well-tolerated by living organisms, other components in its formulation like lecithin and non-ionic emulsifiers, are potentially toxic (Rahman *et al.*, 2013). In this study, non-loaded NLC did not produce GI50, LD50 or cytostatic dose values after 24, 48 or 72 hr of treatment showing that cytotoxic effect of ZER-NLC on CMT-stylo cells is primarily due to ZER and not NLC.

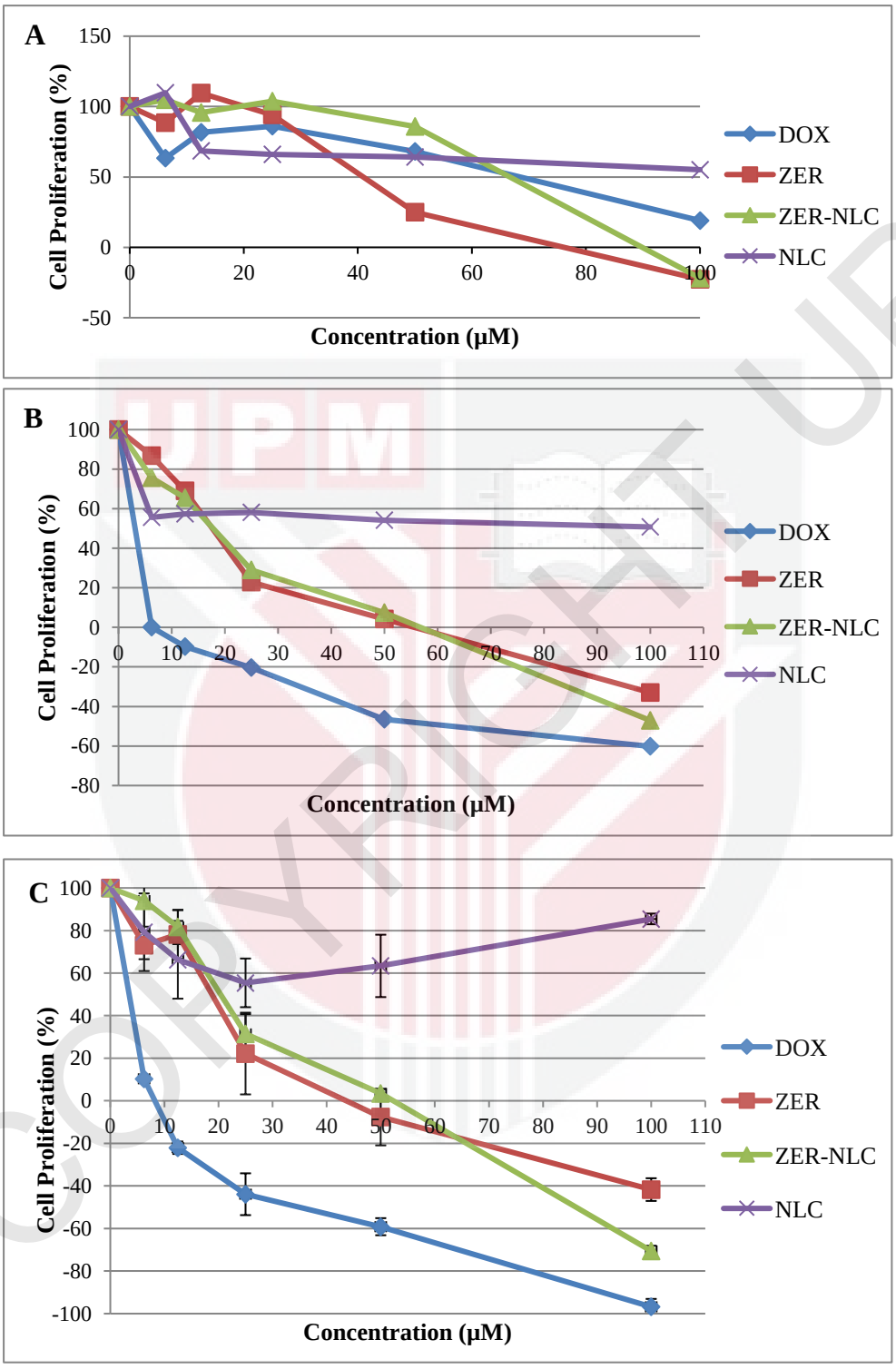

Figure 2: Cell proliferation of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier and free Nanostructured Lipid Carrier at A) 24 hr, B) 48 hr and C) 72 hr.

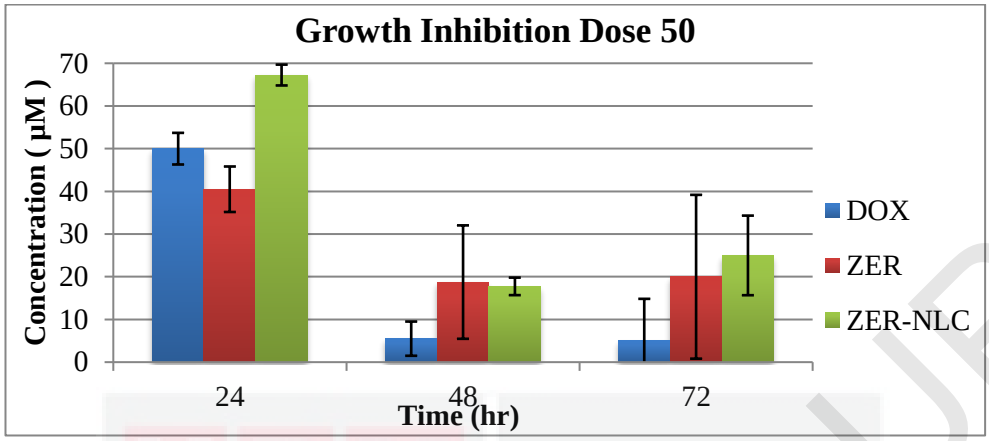

Figure 3: Growth Inhibition Dose 50 (GI50) of Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) on CMT-stylo cells at 24, 48 and 72 hr.

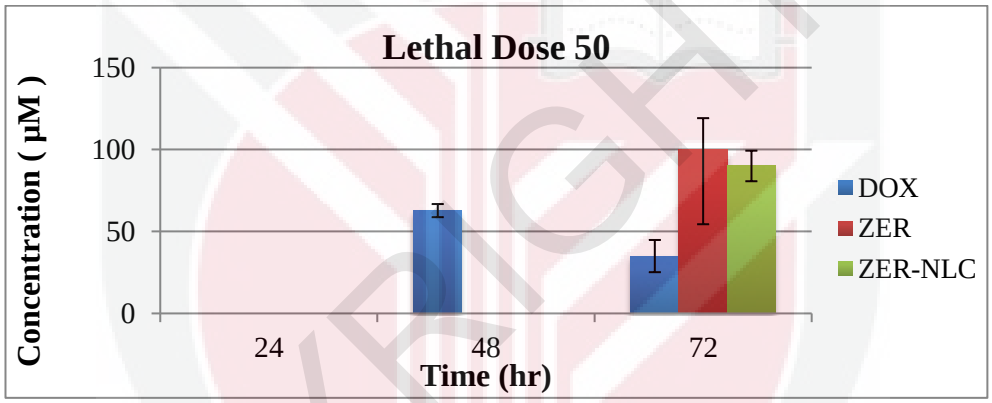

Figure 4: Lethal Dose 50 (LD50) Of Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) on CMT-stylo cells at 24, 48 and 72 hr.

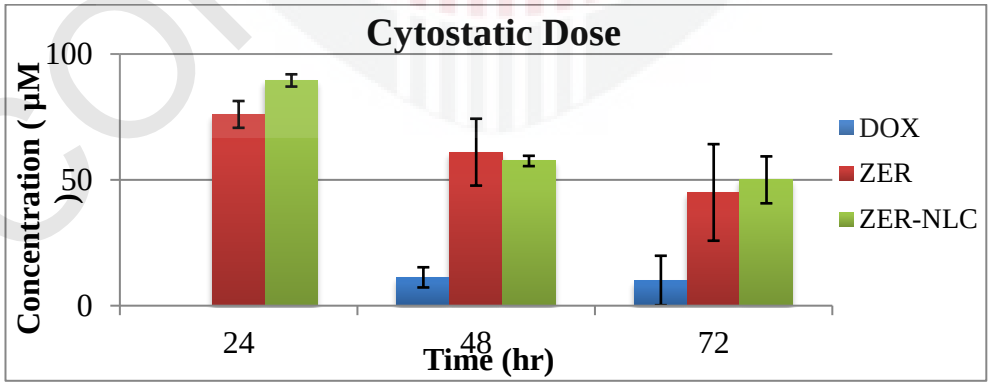

Figure 5: Cytostatic concentration of Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) on CMT-stylo cells at 24, 48 and 72 hr.

4.2 Cell Morphology Imaging

4.2.1 Inverted Light Microscopy

In this study, the morphological changes in CMT-stylo cells after treatment with ZER, ZER-NLC and NLC were determined by inverted light microscopy at 24, 48 and 72 hr of treatment (Figures 6, 7 and 8).

Cells treated with ZER showed typical apoptotic morphological changes like cell membrane blebbing, cell shrinkage, vacuole formation, chromatin condensation and nuclear fragmentation after 24, 48 and 72 hr of treatment (Lin *et al*., 2007; Doonan and Cotter, 2008). Late apoptotic changes like apoptotic bodies' formation and chromatin cleavage were seen after 72 hr of ZER treatment (Figure 8).

As for cells treated with ZER-NLC, apoptotic changes similar to ZER treated cells were only prominent after 48 and 72 hr of treatments. At 24 hr of ZER-NLC treatment, the majority of CMT-stylo cells are still showing normal cell morphology with minimal vacuolisation. This finding corresponds with other findings in this study that showed ZER-NLC produced slower and more controlled cytotoxicity on CMT-stylo cells than pure ZER. However after 48 and 72 hr of treatment, ZER and ZER-NLC treated cells showed similar apoptotic changes. Therefore, it can be deduced

that incorporation of ZER into NLC provides the much desired controlled release characteristic of a drug carrier without altering the anticancer activity of the loaded ZER. This finding is similar to that shown by Rahman *et al.*, (2013; 2014) on human T-cell acute lymphoblastic leukemia (Jurkat) cells. As for NLC-treated cells, there was no significant apoptotic change after 24, 48 and 72 hr of treatment.

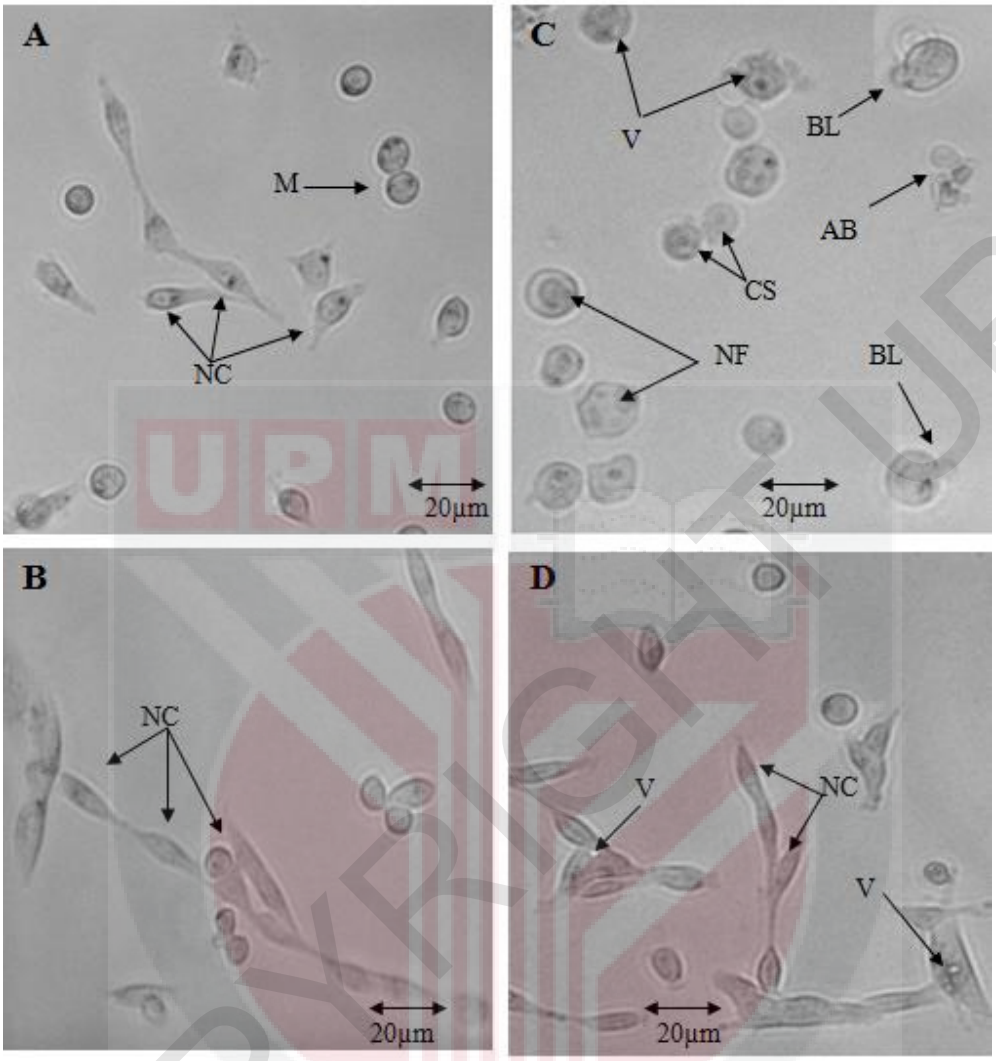

Figure 6: Morphology of CMT-stylo cells treated with Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (NLC) (ZER-NLC) and NLC after 24 hr.

(A) Untreated cells showing normal cell morphology and mitosis. (B) NLC-treated cells showing normal morphology. (C) ZER-treated cells showing cell membrane blebbing, cell shrinkage, vacuole formation and nuclear fragmentation. Apoptotic bodies were evident. (D) ZER-NLC treated cells with the majority remained generally normal, although a few cells showed vacuolization.

[NC= normal cells; M=mitosis; CS=cell shrinkage; BL=cell membrane blebbing; AB=apoptotic body; NF=nuclear fragmentation; V=vacuolisation]

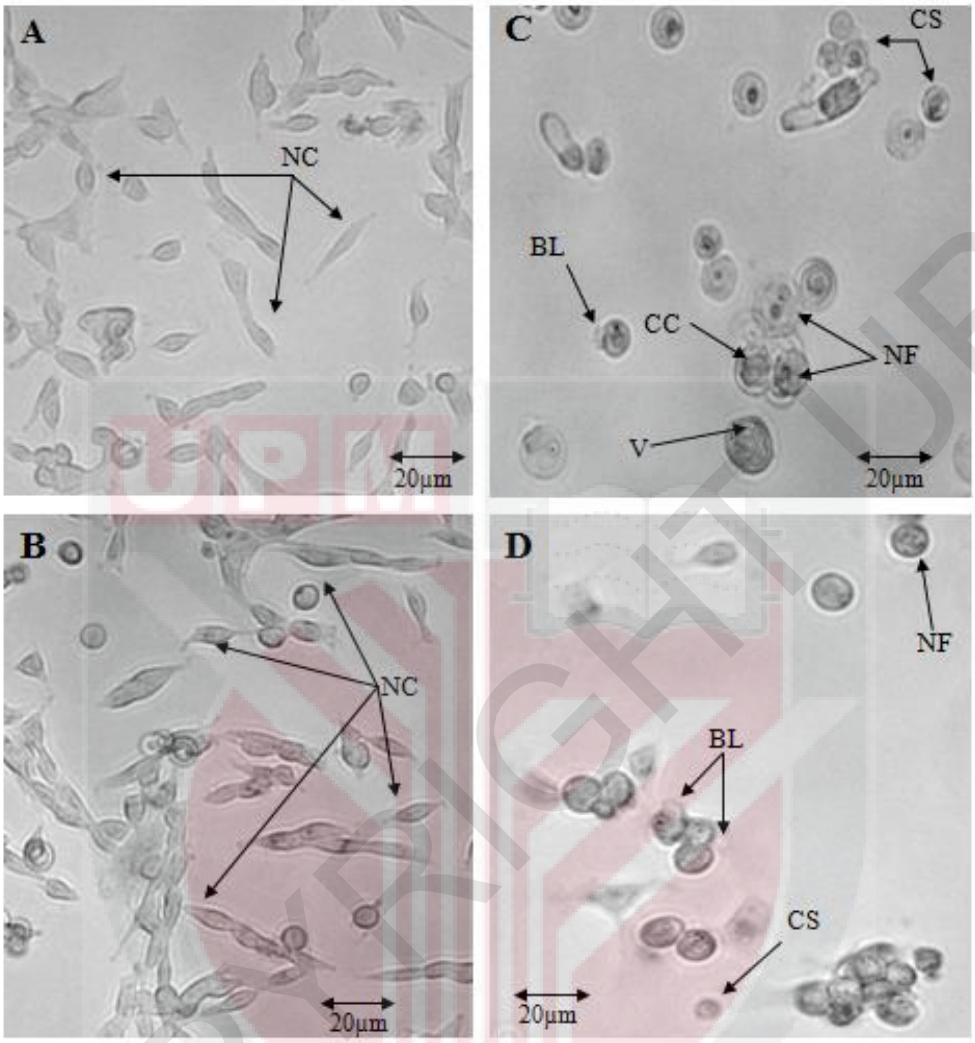

Figure 7: Morphology of CMT-stylo cells treated Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (NLC) (ZER-NLC) and NLC after 48 hr.

(A) Untreated cells and (B) NLC-treated cells showing normal morphology. (C) ZER-treated cells showing cell membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and vacuole formation. (D) ZER-NLC treated cells showing cell membrane blebbing, cell shrinkage and nuclear fragmentation.

[NC= normal cells; M=mitosis; CS=cell shrinkage; BL=cell membrane blebbing; AB=apoptotic body; NF=nuclear fragmentation; V=vacuolisation]

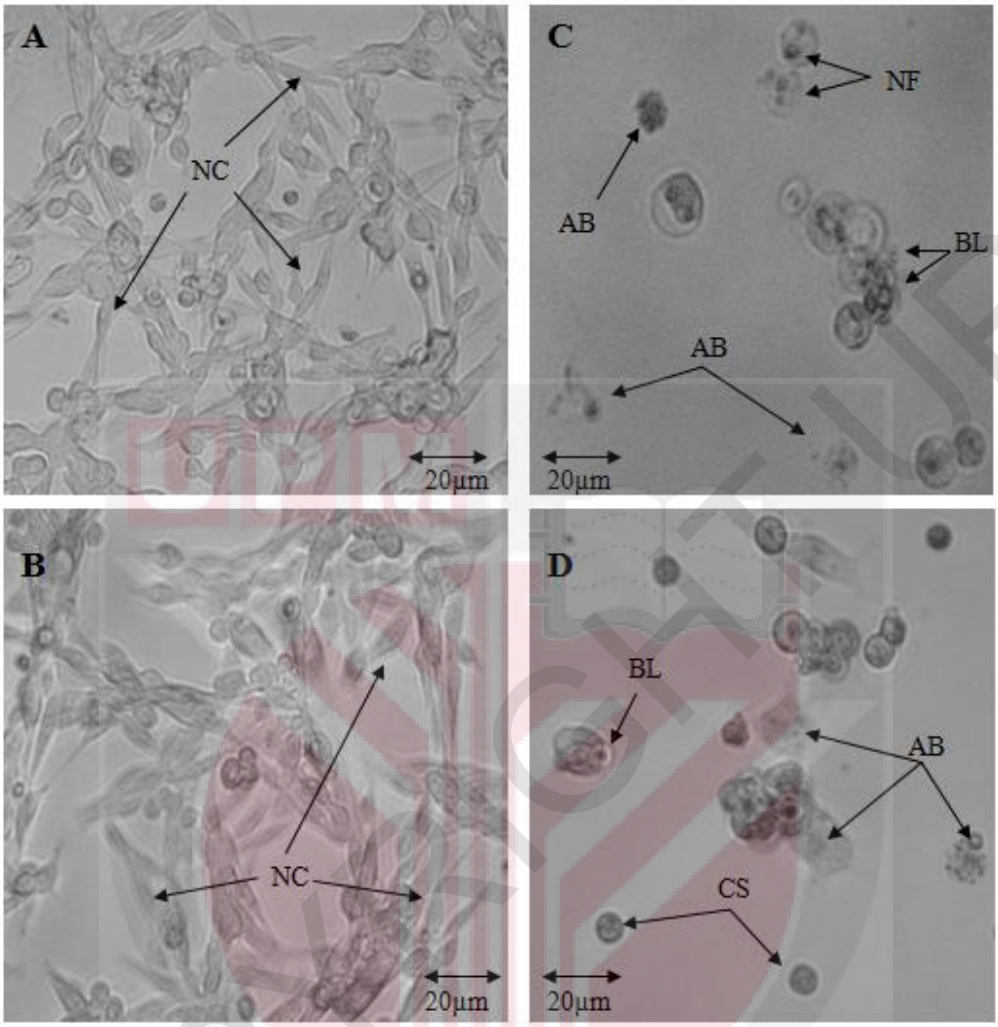

Figure 8: Morphology of CMT-stylo cells treated with Zerumbone (ZER), ZER-loaded nanostructured lipid carrier (NLC) (ZER-NLC) and NLC after 72 hr.

(A) Untreated cells and (B) NLC-treated cells showing normal cell morphology. (C) ZER-treated cells showing cell membrane blebbing, nuclear fragmentation and apoptotic bodies. (D) ZER-NLC treated cells showing cell membrane blebbing, cell shrinkage and apoptotic bodies.

[NC= normal cells; CS=cell shrinkage; BL=cell membrane blebbing; AB=apoptotic body.]

4.2.2 Acridine Orange and Propridium Iodide Staining

To confirm the cytotoxic effect of ZER and ZER-NLC, the CMT-stylo cells were subjected to acridine orange (AO) and propridium iodide (PI) double staining and examined under fluorescence light microscopy (Ribble *et al*., 2005).

The hallmark of apoptosis like cell membrane blebbing, nucleus margination and chromatin condensation were seen in both ZER- and ZER-NLC-treated cells after 72 hr (Figure 9). Late apoptotic cells showing orange-red nucleus and greenish chromatin condensation were typical of CMT-stylo cells treated with ZER and ZER-NLC. On the other hand, untreated viable cells appeared intact and oval-shaped with greenish appearance. Similar viable CMT-stylo cells were also seen after NLC treatment. Nanostructured lipid carrier did not cause apoptosis of CMT-stylo cells. Therefore, this study showed that it is the ZER in ZER-NLC that is responsible for CMT-stylo cell apoptosis.

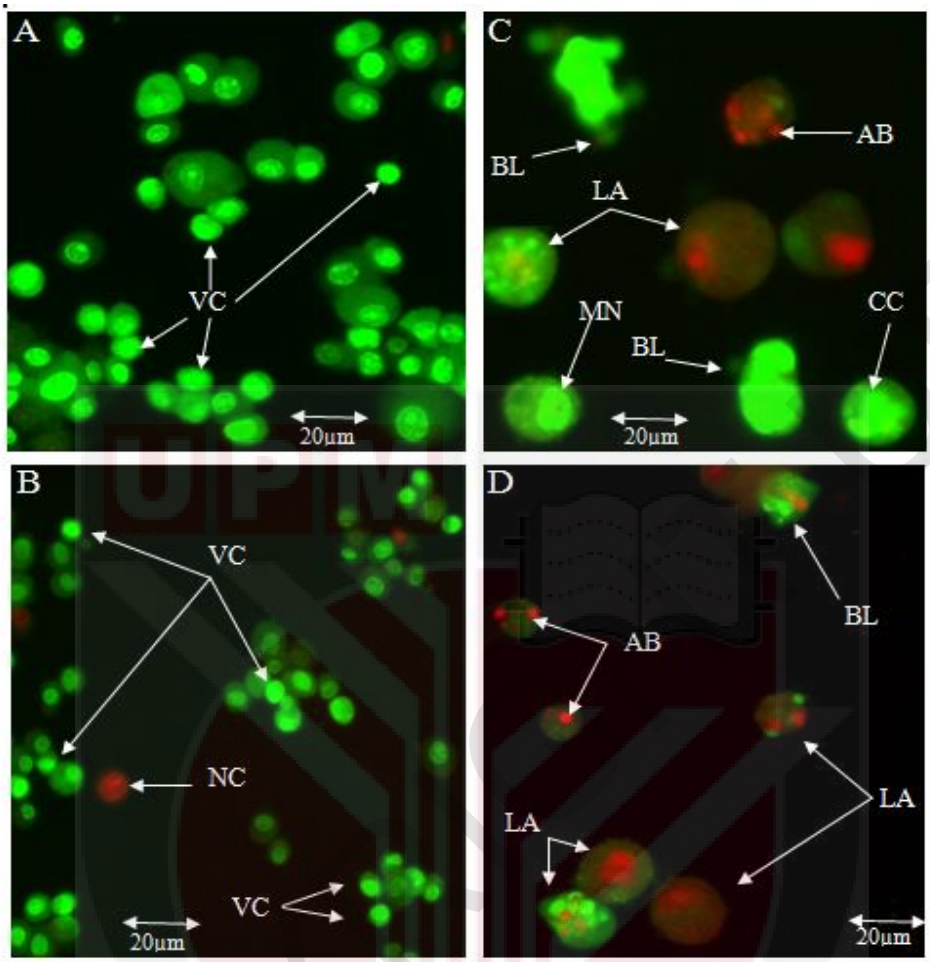

Figure 9: Fluorescence micrograph of CMT-stylo cells treated with Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (NLC) (ZER-NLC) and NLC doubled stained with acridine orange (AO) and propridium iodide (PI) after 72 hr.

(**A**) Untreated cells showing a normal structure without significant apoptosis or necrosis. (**B**) Intact NLC treated cells showing fluorescent greenish staining. Necrotic cells displayed a reddish nucleus with an intact structure. (**C**) ZER-treated cells with early apoptotic cells showing intercalated bright green staining with a marginated nucleus, chromatin condensation and blebbing. Late apoptosis and apoptotic bodies were also evident. (**D**) ZER-NLC treated cells showing blebbing, late apoptosis and apoptotic bodies. (200× magnification).

[VC=viable cells; EA=early apoptotic cells; CC=chromatin condensation; MN=marginated nucleus; BL=cell membrane blebbing; AB=apoptotic body; LA=late apoptotic cells; NC=necrotic cell.]

Apoptosis, the programmed cell death, is an active process. It is a normal component of the development and health of multicellular organisms. In oncological studies, apoptosis is important because failure in the apoptotic mechanism is one of the causes of cancer (Ribble *et al*., 2005). Apoptosis, a desired biodefense cell death can be induced in cancer tissues (Kang *et al*., 2007). This induction is found to be possible via the use of natural products from a variety of plants-derived compounds (Matito *et al*., 2003). Zerumbone, a major compound from the essential volatile oil of edible wild ginger rhizomes, *Zingiber zerumbet* (L.) Smith has been previously reported to induce apoptosis in several cancer cell lines, such as liver cancer (Sharifah Sakinah *et al*., 2007), leukemia (Jurkat) (Abdelwahab *et al*., 2010) and cervical cancer (HeLa) (Abdelwahab *et al*., 2009) cells.

In this study, we found that cytotoxicity of pure ZER and ZER-NLC on CMT-stylo cells is via apoptosis. This finding confirms the findings of the anticancer effect of ZER-NLC, in previous studies, on a human Jurkat cell line (Rahman *et al.*, 2013; 2014). Since typical apoptotic changes were found in both ZER and ZER-NLC treated cells, we can deduce that incorporation of ZER into NLC does not compromise ZER anticancer effect. It can be concluded that ZER-NLC is an effective drug carrier system for the treatment of cancers.

5.0 CONCLUSION

This study, for the first time, provides evidences that ZER-NLC is a potentially effective drug delivery system for the treatment of canine mammary gland tumours. The anticancer effect of ZER-NLC on CMT-stylo cell line occurs via induction of apoptosis. The study also showed that incorporation of ZER into NLC did not alter its anticancer effect. Therefore, ZER-NLC, having promising anticancer effect, can be developed into a novel, innovative and relatively cheap drug-carrier system for a safe treatment of cancers.

6.0 FUTURE STUDIES

It is recommended *in vivo* studies using ZER-NLC onto canine mammary gland tumour patient be carried out. The intratumoural application of ZER-NLC is an area that should be pursued in the future to realise the full anticancer effect of ZER-NLC.

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8.0 APPENDIX

Appendix 1: Schematic of 96 well plates of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC).

Plate 1:

		DOX					ZER					
	1	2	3	4	5	6	7	8	9	10	11	12
A	x	x	x	x	x	x	x	x	x	x	x	x
B	x											x
C	x											x
D	x											x
E	x											x
F	x											x
G	x											x
H	x	x	x	x	x	x	x	x	x	x	x	x

Plate 2:

		ZER-NLC					NLC					
	1	2	3	4	5	6	7	8	9	10	11	12
A	x	x	x	x	x	x	x	x	x	x	x	x
B	x											x
C	x											x
D	x											x
E	x											x
F	x											x
G	x											x
H	x	x	x	x	x	x	x	x	x	x	x	x

*each well was seeded with 5000 CMT-stylo cells.

Legend:

B = Cells + 100µM of treatment
C = Cells + 50µM of treatment
D = Cells + 25µM of treatment
E = Cells + 12.5µM of treatment
F = Cells + 6.5µM of treatment
G = Cells + 0µM of treatment (Negative control group)
X = Well not used

Appendix 2: Schematic of 6 well plates of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC).

*each well was seeded with 50000 CMT-stylo cells.

Plate 1:

	1	2	3
A			
B			

Legend

A = Cells + DOX with LD50, Cytostatic Dose and GI50 respectively (Positive control)

B = Cells + ZER with LD50, Cytostatic Dose and GI50 respectively

Plate 2:

	1	2	3
A			
B			X

Legend

A = Cells + ZER-NLC with LD50, Cytostatic Dose and GI50 respectively

B = Cells + NLC with LD50 and GI50 respectively

X = Cells without treatment (Negative control group)

Appendix 3: Values of LD50, Cytostatic Dose and GI50 for Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC).

Concentration(μM)	Treatments			
	DOX	ZER	ZER-NLC	NLC
LD50	35	100	90	90
Cytostatic	10	45	50	-
GI50	5	20	25	25

*Since NLC did not have any LD50, Cytostatic Dose and GI50 even after 72hr of treatment; thus, the value LD50 and GI50 of ZER-NLC were used for NLC.

Appendix 4: Statistical analyses of data for cell proliferation of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC) at 24 hr.

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_Proliferation _24hr	DOX	.104	30	.200*	.972	30	.600
	ZER	.173	30	.022	.931	30	.052
	ZER-NLC	.197	30	.004	.903	30	.010
	NLC	.119	30	.200*	.953	30	.203

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Between-Subjects Effects (ANOVA)

Dependent Variable: Cell_ Proliferation _24hr

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	231701.712[a]	23	10073.987	11.510	.000
Intercept	704225.344	1	704225.344	804.577	.000
Treatment	27957.598	3	9319.199	10.647	.000
Dose	133319.583	5	26663.917	30.464	.000
Treatment * Dose	70424.531	15	4694.969	5.364	.000
Error	84026.259	96	875.274		
Total	1019953.315	120			
Corrected Total	315727.971	119			

a. R Squared = .734 (Adjusted R Squared = .670)

Pairwise Comparisons

Dependent Variable: Cell_ Proliferation _24hr

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
DOX	ZER	5.675	7.639	.459	-9.488	20.838
	ZER-NLC	-32.791*	7.639	.000	-47.954	-17.628
	NLC	.590	7.639	.939	-14.573	15.753
ZER	DOX	-5.675	7.639	.459	-20.838	9.488
	ZER-NLC	-38.466*	7.639	.000	-53.629	-23.303
	NLC	-5.085	7.639	.507	-20.248	10.078
ZER-NLC	DOX	32.791*	7.639	.000	17.628	47.954
	ZER	38.466*	7.639	.000	23.303	53.629
	NLC	33.382*	7.639	.000	18.219	48.545
NLC	DOX	-.590	7.639	.939	-15.753	14.573
	ZER	5.085	7.639	.507	-10.078	20.248
	ZER-NLC	-33.382*	7.639	.000	-48.545	-18.219

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Cell_Proliferation_24hr

Tukey HSD

Treatment	N	Subset	
		1	2
ZER	30	64.2999	
NLC	30	69.3847	
DOX	30	69.9749	
ZER-NLC	30		102.7663
Sig.		.879	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 875.274.

a. Uses Harmonic Mean Sample Size = 30.000.

b. Alpha = .05.

Tests of Normality[c]

	Dose	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_Proliferation _24hr	100	.220	20	.012	.871	20	.012
	50	.152	20	.200*	.883	20	.020
	25	.119	20	.200*	.972	20	.798
	12.5	.125	20	.200*	.884	20	.021
	6.25	.113	20	.200*	.964	20	.619

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

c. Cell_Proliferation_24hr is constant when Dose = 0. It has been omitted.

Pairwise Comparisons

Dependent Variable: Cell_ Proliferation _24hr

(I) Dose	(J) Dose	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
100	50	-58.078*	9.356	.000	-76.649	-39.507
	25	-84.545*	9.356	.000	-103.116	-65.975
	12.5	-97.209*	9.356	.000	-115.779	-78.638
	6.25	-82.833*	9.356	.000	-101.404	-64.263
	0	-92.605*	9.356	.000	-111.176	-74.035
50	100	58.078*	9.356	.000	39.507	76.649
	25	-26.467*	9.356	.006	-45.038	-7.896
	12.5	-39.130*	9.356	.000	-57.701	-20.560
	6.25	-24.755*	9.356	.010	-43.326	-6.184
	0	-34.527*	9.356	.000	-53.098	-15.956
25	100	84.545*	9.356	.000	65.975	103.116
	50	26.467*	9.356	.006	7.896	45.038
	12.5	-12.663	9.356	.179	-31.234	5.908
	6.25	1.712	9.356	.855	-16.859	20.283
	0	-8.060	9.356	.391	-26.631	10.511
12.5	100	97.209*	9.356	.000	78.638	115.779
	50	39.130*	9.356	.000	20.560	57.701
	25	12.663	9.356	.179	-5.908	31.234
	6.25	14.375	9.356	.128	-4.196	32.946
	0	4.603	9.356	.624	-13.968	23.174
6.25	100	82.833*	9.356	.000	64.263	101.404
	50	24.755*	9.356	.010	6.184	43.326
	25	-1.712	9.356	.855	-20.283	16.859
	12.5	-14.375	9.356	.128	-32.946	4.196
	0	-9.772	9.356	.299	-28.343	8.799
0	100	92.605*	9.356	.000	74.035	111.176
	50	34.527*	9.356	.000	15.956	53.098
	25	8.060	9.356	.391	-10.511	26.631

12.5	-4.603	9.356	.624	-23.174	13.968
6.25	9.772	9.356	.299	-8.799	28.343

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Cell_Proliferation_24hr

Tukey HSD

Dose	N	Subset		
		1	2	3
100	20	7.3946		
50	20		65.4728	
6.25	20		90.2280	90.2280
25	20		91.9400	91.9400
0	20			100.0000
12.5	20			104.6032
Sig.		1.000	.061	.642

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 875.274.

a. Uses Harmonic Mean Sample Size = 20.000.

b. Alpha = .05.

Appendix 5: Statistical analyses of data for cell proliferation of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC) at 48hr.

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_ Proliferation _48hr	DOX	.284	30	.000	.751	30	.000
	ZER	.169	30	.028	.889	30	.005
	ZER-NLC	.134	30	.180	.908	30	.013
	NLC	.273	30	.000	.749	30	.000

a. Lilliefors Significance Correction

Tests of Between-Subjects Effects (ANOVA)

Dependent Variable: Cell_ Proliferation _48hr

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	317290.836[a]	23	13795.254	265.656	.000
Intercept	127726.126	1	127726.126	2459.633	.000
Treatment	85689.954	3	28563.318	550.046	.000
Dose	176673.320	5	35334.664	680.443	.000
Treatment * Dose	54927.563	15	3661.838	70.516	.000
Error	4985.177	96	51.929		
Total	450002.139	120			
Corrected Total	322276.013	119			

a. R Squared = .985 (Adjusted R Squared = .981)

Pairwise Comparisons

Dependent Variable: Cell_ Proliferation _48hr

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
DOX	ZER	-53.232*	1.861	.000	-56.925	-49.539
	ZER-NLC	-49.584*	1.861	.000	-53.277	-45.891
	NLC	-72.357*	1.861	.000	-76.050	-68.663
ZER	DOX	53.232*	1.861	.000	49.539	56.925
	ZER-NLC	3.648	1.861	.053	-.045	7.341
	NLC	-19.125*	1.861	.000	-22.818	-15.431
ZER-NLC	DOX	49.584*	1.861	.000	45.891	53.277
	ZER	-3.648	1.861	.053	-7.341	.045
	NLC	-22.773*	1.861	.000	-26.466	-19.079
NLC	DOX	72.357*	1.861	.000	68.663	76.050
	ZER	19.125*	1.861	.000	15.431	22.818
	ZER-NLC	22.773*	1.861	.000	19.079	26.466

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Cell_Proliferation_48hr

Tukey HSD

Treatment	N	Subset		
		1	2	3
DOX	30	-11.1682		
ZER-NLC	30		38.4157	
ZER	30		42.0637	
NLC	30			61.1884
Sig.		1.000	.210	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 51.929.

a. Uses Harmonic Mean Sample Size = 30.000.

b. Alpha = .05.

Tests of Normality[c]

	Dose	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_Proliferation_48hr	100	.270	20	.000	.763	20	.000
	50	.230	20	.007	.904	20	.048
	25	.141	20	.200*	.916	20	.083
	12.5	.265	20	.001	.841	20	.004
	6.25	.276	20	.000	.802	20	.001

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

c. Cell_Proliferation_48hr is constant when Dose = 0. It has been omitted.

Pairwise Comparisons

Dependent Variable: Cell_Proliferation_48hr

(I) Dose	(J) Dose	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
100	50	-26.492*	2.279	.000	-31.015	-21.969
	25	-44.752*	2.279	.000	-49.275	-40.228
	12.5	-66.629*	2.279	.000	-71.152	-62.106
	6.25	-69.729*	2.279	.000	-74.252	-65.205
	0	-122.370*	2.279	.000	-126.894	-117.847
50	100	26.492*	2.279	.000	21.969	31.015
	25	-18.260*	2.279	.000	-22.783	-13.736
	12.5	-40.137*	2.279	.000	-44.660	-35.613
	6.25	-43.237*	2.279	.000	-47.760	-38.713
	0	-95.878*	2.279	.000	-100.402	-91.355
25	100	44.752*	2.279	.000	40.228	49.275
	50	18.260*	2.279	.000	13.736	22.783
	12.5	-21.877*	2.279	.000	-26.401	-17.354
	6.25	-24.977*	2.279	.000	-29.500	-20.454
	0	-77.619*	2.279	.000	-82.142	-73.095
12.5	100	66.629*	2.279	.000	62.106	71.152
	50	40.137*	2.279	.000	35.613	44.660
	25	21.877*	2.279	.000	17.354	26.401

6.25	6.25	-3.100	2.279	.177	-7.623	1.423
	0	-55.742*	2.279	.000	-60.265	-51.218
	100	69.729*	2.279	.000	65.205	74.252
	50	43.237*	2.279	.000	38.713	47.760
	25	24.977*	2.279	.000	20.454	29.500
	12.5	3.100	2.279	.177	-1.423	7.623
	0	-52.642*	2.279	.000	-57.165	-48.118
	100	122.370*	2.279	.000	117.847	126.894
	50	95.878*	2.279	.000	91.355	100.402
0	25	77.619*	2.279	.000	73.095	82.142
	12.5	55.742*	2.279	.000	51.218	60.265
	6.25	52.642*	2.279	.000	48.118	57.165

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Cell_Proliferation_48hr

Tukey HSD

Dose	N	Subset				
		1	2	3	4	5
100	20	-22.3704				
50	20		4.1217			
25	20			22.3812		
12.5	20				44.2585	
6.25	20				47.3584	
0	20					100.0000
Sig.		1.000	1.000	1.000	.750	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 51.929.

a. Uses Harmonic Mean Sample Size = 20.000.

b. Alpha = .05.

Appendix 6: Statistical analyses of data for cell proliferation of CMT-stylo cells treated with Doxorubicin (DOX), Zerumbone (ZER), ZER-loaded Nanostructured Lipid Carrier (ZER-NLC) and free Nanostructured Lipid Carrier (NLC) at 72 hr.

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_Proliferation _72hr	DOX	.161	30	.046	.878	30	.002
	ZER	.152	30	.077	.926	30	.038
	ZER-NLC	.173	30	.022	.875	30	.002
	NLC	.119	30	.200*	.931	30	.053

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Between-Subjects Effects (ANOVA)

Dependent Variable: Cell_ Proliferation _72hr

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	457677.442[a]	23	19899.019	141.232	.000
Intercept	134151.262	1	134151.262	952.129	.000
Treatment	134245.000	3	44748.333	317.598	.000
Dose	229272.455	5	45854.491	325.449	.000
Treatment * Dose	94159.988	15	6277.333	44.553	.000
Error	13526.026	96	140.896		
Total	605354.730	120			
Corrected Total	471203.468	119			

a. R Squared = .971 (Adjusted R Squared = .964)

Pairwise Comparisons

Dependent Variable: Cell_ Proliferation _72hr

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
DOX	ZER	-56.909*	3.065	.000	-62.993	-50.825
	ZER-NLC	-60.229*	3.065	.000	-66.313	-54.145
	NLC	-92.990*	3.065	.000	-99.073	-86.906
ZER	DOX	56.909*	3.065	.000	50.825	62.993
	ZER-NLC	-3.320	3.065	.281	-9.404	2.764
	NLC	-36.081*	3.065	.000	-42.164	-29.997
ZER-NLC	DOX	60.229*	3.065	.000	54.145	66.313
	ZER	3.320	3.065	.281	-2.764	9.404
	NLC	-32.761*	3.065	.000	-38.844	-26.677
NLC	DOX	92.990*	3.065	.000	86.906	99.073
	ZER	36.081*	3.065	.000	29.997	42.164

ZER-NLC	32.761*	3.065	.000	26.677	38.844

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Cell_ Proliferation _72hr

Tukey HSD

Treatment	N	Subset		
		1	2	3
DOX	30	-19.0965		
ZER	30		37.8126	
ZER-NLC	30		41.1325	
NLC	30			73.8931
Sig.		1.000	.701	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 140.896.

a. Uses Harmonic Mean Sample Size = 30.000.

b. Alpha = .05.

Tests of Normality[c]

	Dose	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Cell_ Proliferation _72hr	100	.262	20	.001	.763	20	.000
	50	.150	20	.200*	.940	20	.244
	25	.184	20	.075	.882	20	.020
	12.5	.219	20	.013	.856	20	.007
	6.25	.176	20	.104	.897	20	.036

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Pairwise Comparisons

Dependent Variable: Cell_ Proliferation _72hr

(I) Dose	(J) Dose	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
100	50	-30.576*	3.754	.000	-38.027	-23.125
	25	-47.674*	3.754	.000	-55.125	-40.223
	12.5	-89.365*	3.754	.000	-96.816	-81.914
	6.25	-91.609*	3.754	.000	-99.060	-84.158
	0	-131.722*	3.754	.000	-139.173	-124.271
50	100	30.576*	3.754	.000	23.125	38.027
	25	-17.098*	3.754	.000	-24.548	-9.647
	12.5	-58.789*	3.754	.000	-66.240	-51.338
	6.25	-61.033*	3.754	.000	-68.484	-53.582
	0	-101.146*	3.754	.000	-108.597	-93.695
25	100	47.674*	3.754	.000	40.223	55.125
	50	17.098*	3.754	.000	9.647	24.548
	12.5	-41.691*	3.754	.000	-49.142	-34.240
	6.25	-43.936*	3.754	.000	-51.386	-36.485
	0	-84.049*	3.754	.000	-91.499	-76.598
12.5	100	89.365*	3.754	.000	81.914	96.816
	50	58.789*	3.754	.000	51.338	66.240
	25	41.691*	3.754	.000	34.240	49.142
	6.25	-2.244	3.754	.551	-9.695	5.207
	0	-42.357*	3.754	.000	-49.808	-34.906
6.25	100	91.609*	3.754	.000	84.158	99.060
	50	61.033*	3.754	.000	53.582	68.484
	25	43.936*	3.754	.000	36.485	51.386
	12.5	2.244	3.754	.551	-5.207	9.695
	0	-40.113*	3.754	.000	-47.564	-32.662
0	100	131.722*	3.754	.000	124.271	139.173
	50	101.146*	3.754	.000	93.695	108.597
	25	84.049*	3.754	.000	76.598	91.499
	12.5	42.357*	3.754	.000	34.906	49.808
	6.25	40.113*	3.754	.000	32.662	47.564

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

c. Cell_ Proliferation _72hr is constant when Dose = 0. It has been omitted.

Cell_ Proliferation _72hr

Tukey HSD

Dose	N	Subset				
		1	2	3	4	5
100	20	-31.7223				
50	20		-1.1462			
25	20			15.9514		
12.5	20				57.6426	
6.25	20				59.8869	
0	20					100.0000
Sig.		1.000	1.000	1.000	.991	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 140.896.

a. Uses Harmonic Mean Sample Size = 20.000.

b. Alpha = .05.