
UNIVERSITI PUTRA MALAYSIA

# HYDROPHOBIC MODIFICATION OF CHITOSAN NANOPARTICLES FOR INCREASED THERAPEUTIC DELIVERY AND ANTICANCER PROPERTIES TOWARDS HUMAN CANCER CELL LINES

CHA YEE KUEN

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**By**

**CHA YEE KUEN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

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**Chairman : Associate Professor Mas Jaffri Masarudin, PhD**
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Lung cancer is the leading cause of cancer-related deaths for men and second for women worldwide, with approximately 1.6 million of deaths reported annually. Conventional delivery of anticancer drugs for treatment is less effective due to intrinsic obstacles such as poor aqueous solubility and poor cellular accumulation of drugs. Additionally, chemotherapy incurs various concomitant undesirable side effects including cytotoxicity, cardiotoxicity and neurotoxicity. Thus, the utilization of natural anticancer therapeutics as an alternative is required to prevent these undesirable consequences. Silibinin (SLB) is a poorly-soluble plant extract derived from *Silybum marianum* which is commonly used for treatment of liver diseases. SLB exhibits anti-cancer properties by inhibiting cell growth, angiogenesis, proliferation, cell migration and inducing apoptotic death. However, poor solubility and cell accumulation of SLB limits the use of this compound as an alternative anticancer therapeutic. Therefore, the employment of nanotechnological-based applications have aided in the advancement of better diagnosis and more efficient treatment of cancer. Additionally, the utilization of nanoparticulate delivery can reduce the dosage of the natural therapeutic compound, reducing potential side effects of therapeutics. Based on the results, a low encapsulation efficiency was achieved by conventional chitosan nanoparticle (CNP) system. This necessitates modification of the CNP through hydrophobic modification to develop a palmitoyl-chitosan nanoparticle (pCNP) system for enhanced encapsulation efficiency and therapeutic efficacy. Various physico-chemical characterization analyses was conducted to evaluate the pCNP system. Utilization of free amine percentage of pCNP increased by 15% due to the conjugation of –NHS palmitic acid to the polymer, in addition to TPP. The pCNP synthesized revealed an expansion in size

and decrease in polydispersity index (PDI) compared to CNP, indicating greater monodispersity in pCNP. The encapsulation of SLB into pCNP showed an expansion in size and PDI, signifying the heterogeneity of nanoparticles after encapsulation and correlating to morphological analyses. Moreover, the encapsulation efficiency improved 1-fold from 25% of CNP-SLB to 50% of pCNP-SLB. Subsequently, MTT cytotoxicity assay showed enhanced efficacy of pCNP-SLB over CNP-SLB and free SLB. It was found that pCNP-SLB was most competitive in A549 cell line compared to SW620 and 786-O cell lines as shown by the lowest $IC_{50}$ value of 7.77 μM among different cancer cell lines and time points. Further study conducted using A549 cell line has demonstrated that pCNP-SLB has enhanced the anti-invasive effect of SLB through cell migration assay and suggested the synergistic effect between pCNP and SLB. On the other hand, Annexin V apoptosis assay and cell cycle analysis have also shown that pCNP-SLB revealed a correlated results of enhanced apoptotic death and G1 cell cycle arrest in A549 cells with sustained release properties. These results have therefore revealed the enhanced therapeutic efficacy and sustained release properties of pCNP-SLB which required low dosing to achieve high therapeutic efficacy. Additionally, subsequent study on pCNP encapsulation on protocatechuic acid (PCA) has revealed that pCNP can be utilized to other poorly soluble compounds other than SLB. Conclusively, various physico-chemical analyses have indicated the modification on CNP system and signified the successful encapsulation of SLB into pCNP. Besides, the *in vitro* cellular study has suggested an enhanced therapeutic efficacy of pCNP-SLB compared to conventional CNP-SLB and free SLB towards A549 cells with sustained release properties. This system can potentially be developed into novel delivery system for many other hydrophobic therapeutics to aid in pharmaceutical and biomedical fields.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENGUBAHSUAIAN HIDROFOBIK NANOPARTIKEL KITOSAN UNTUK MEMPERTINGKATKAN PENGHANTARAN TERAPEUTIK DAN CIRI-CIRI ANTIKANSER TERHADAP TITISAN SEL-SEL KANSER**

Oleh

**CHA YEE KUEN**

**Julai 2020**

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Kanser paru-paru adalah penyebab utama kematian yang berkaitan dengan barah untuk lelaki dan kedua untuk wanita di seluruh dunia, dengan kira-kira 1.6 juta kematian tahunan dilaporkan dan 1.8 juta kes baru ditemui setiap tahun. Penyampaian ubat antikanker secara konvensional menjadi kurang berkesan kerana rintangan intrinsik seperti kelarutan berair yang buruk dan pengumpulan ubat sel yang lemah. Selain itu, kemoterapi menimbulkan pelbagai kesan sampingan yang tidak diingini termasuk sitotoksisitas, kardiotoksisiti dan neurotoksisiti. Oleh itu, penggunaan terapi antikanker semula jadi sebagai alternatif diperlukan untuk mengelakkan akibat yang tidak diingini ini. Silibinin (SLB) adalah ekstrak tumbuhan yang kurang larut yang berasal dari *Silybum marianum* yang biasanya digunakan untuk perawatan penyakit hati. Telah ditunjukkan bahawa dalam kajian baru-baru ini, SLB menunjukkan sifat anti-kanser dengan menghalang pertumbuhan sel, angiogenesis, percambahan, migrasi sel dan menyebabkan kematian apoptosis. Walau bagaimanapun, kelarutan dan pengumpulan sel SLB yang lemah telah membatasi penggunaan sebatian semula jadi ini sebagai terapi antikanker semula jadi alternatif. Oleh itu, penggunaan aplikasi berasaskan nanoteknologi telah membantu kemajuan diagnosis yang lebih baik dan rawatan penyakit yang lebih cekap, termasuk barah. Selain itu, penggunaan penghantaran nanopartikulat dapat mengurangkan dos sebatian terapi semula jadi, di mana dapat mengurangkan kemungkinan kesan sampingan terapi. Berdasarkan hasilnya, peratusan kecekapan enkapsulasi rendah dicapai oleh sistem nanopartikel kitosan konvensional (CNP). Ini telah menunjukkan keperluan untuk memodifikasi sistem CNP ini melalui modifikasi hidrofobik dengan menggunakan NHS-palmitic acid untuk menghasilkan sistem nanopartikel palmitoyl-kitosan (pCNP) untuk peningkatan kecekapan enkapsulasi

dan keberkesanan terapi. Pelbagai analisis pencirian fiziko-kimia telah dilakukan untuk menilai sistem pCNP. Didapati bahawa penggunaan peratusan amina bebas pCNP meningkat sekitar 15% disebabkan oleh konjugasi –NHS asid palmitik ke polimer sebagai tambahan kepada TPP. PCNP yang disintesis menggunakan kaedah persiapan yang sama telah menunjukkan pengembangan dalam ukuran dan penurunan dalam indeks polidispersi (PDI) dibandingkan dengan CNP, yang menunjukkan monodispersity yang lebih besar dalam pCNP. Enkapsulasi SLB ke dalam pCNP telah menunjukkan pengembangan dalam ukuran dan kenaikan PDI, menandakan heterogenitas nanopartikel setelah enkapsulasi dan berkorelasi sesuai dengan analisis morfologi. Lebih-lebih lagi, kecekapan enkapsulasi telah meningkat sekitar 1 kali ganda dari 25% CNP-SLB menjadi 50% pCNP-SLB. Uji sitotoksisitas MTT yang seterusnya menunjukkan keberkesanan pCNP-SLB yang lebih tinggi berbanding CNP-SLB dan SLB percuma. Didapati bahawa pCNP-SLB paling kompetitif dalam sel sel A549 berbanding dengan garis sel SW620 dan 786-O seperti yang ditunjukkan oleh nilai $IC_{50}$ terendah 7.77 μM di antara garis sel kanser dan titik waktu yang berbeza. Kajian lebih lanjut yang dilakukan menggunakan garis sel A549 telah menunjukkan bahawa pCNP-SLB telah meningkatkan kesan anti-invasif SLB melalui ujian migrasi sel dan menyarankan kesan sinergis antara pCNP dan SLB. Sebaliknya, analisis apoptosis Annexin V dan analisis kitaran sel juga menunjukkan bahawa pCNP-SLB mendedahkan hasil yang berkaitan dengan peningkatan kematian apoptosis dan penangkapan kitaran sel G1 pada sel A549 dengan sifat pelepasan berkelanjutan. Oleh itu, hasil ini telah menunjukkan peningkatan keberkesanan terapi dan sifat pelepasan berkelanjutan pCNP-SLB yang memerlukan dos rendah untuk mencapai keberkesanan terapi yang tinggi. Selain itu, kajian seterusnya mengenai enkapsulasi pCNP pada asam protocatechuic (PCA) telah menunjukkan bahawa pCNP dapat digunakan untuk sebatian lain yang kurang larut selain SLB. Kesimpulannya, pelbagai analisis fisiko-kimia telah menunjukkan pengubahsuaian pada sistem CNP dan menandakan kejayaan enkapsulasi SLB ke dalam pCNP. Selain itu, kajian sel *in vitro* telah menunjukkan peningkatan keberkesanan terapi pCNP-SLB berbanding dengan CNP-SLB konvensional dan SLB bebas terhadap sel A549 dengan sifat pelepasan berterusan. Sistem ini berpotensi dikembangkan menjadi sistem penyampaian novel untuk banyak terapi hidrofobik lain untuk membantu bidang farmaseutikal dan bioperubatan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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| | |
|---|---|
| 786-0 | Adenocarcinomic human renal epithelial cell line |
| A549 | Adenocarcinomic human alveolar basal epithelial cell line |
| $A_{288nm}$ | Absorbance at 288nm |
| $A_{405nm}$ | Absorbance at 405nm |
| $A_{570nm}$ | Absorbance at 570nm |
| ATR-FTIR | Attenuated Total Reflection-Fourier Transform Infrared spectroscopy |
| CNP | Chitosan nanoparticle |
| CNP-SLB | Silibinin encapsulated chitosan nanoparticle |
| CNT | Carbon Nanotube |
| Conc. | Concentration |
| CS | Chitosan |
| CVD | cardiovascular disease |
| $dH_2O$ | Deionized distilled water |
| DLS | Dynamic light scattering |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| E.E | Encapsulation Efficiency |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Feta bovine serum |
| FDA | Food and Drug administration |
| FESEM | Field emmision-scanning electron microscopy |
| FITC | Fluorescein isothiocyanate |
| HCl | Hydrochloric acid |
| $IC_{50}$ | Half maximal inhibitory concentration |
| MRC-5 | Human normal lung fibroblast cell line |
| MTT | 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide |
| MW | Molecular weight |
| NaOH | Sodium hydroxide |
| $NH_2$ | Amine |
| NHS | N-hydroxysuccinimide ester group |
| NSCLC | Non-small cell lung carcinoma |
| PBS | Phosphate buffer saline |
| PCA | Protocatechuic acid |
| pCNP | Palmitoyl chitosan nanoparticle |
| pCNP-SLB | Silibinin encapsulated palmitoyl chitosan nanoparticle |
| pCS | Palmitoyl chitosan |
| PDI | Polydispersity index |
| PEG | Polyethylene glycol |
| PI | Propidium Iodide |
| PSD | Particle size distribution |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPMI | Roswell Park Memorial Institute |

| | |
|---|---|
| SCLC | Small cell lung carcinoma |
| SDS | Sodium dodecyl sulfate |
| SEM | Scanning electron microscopy |
| SLB | Silibinin |
| SW620 | adenocarcinomic human colorectal epithelial cell line |
| TEM | Transmission electron microscopy |
| TNBS | 2,4,6-Trinitrobenzenesulfonic acid |
| TPP | Sodium tripolyphosphate |
| v/v | Volume per volume |
| w/v | Weight per volume |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of study

Over the last decade, the incidence and mortality rate of cancer remained the top leading cause of death worldwide (Kooti *et al*., 2017). Researchers have formulated several means such as surgery, radiation, and chemotherapy to treat cancer patients, but chemotherapy remains the most significant treatment among these approaches, with synthetic drugs such as anthracyclines being the most potent chemotherapeutic drugs used. The four most prominent anthracyclines are epirubicin, doxorubicin, idarubicin and daunorubicin (McGowan *et al*., 2017). Yet, the severe side effects following the chemotherapy due to non-specific destruction have become the main constrain for the treatment including cardiotoxicity, nausea, and alopecia (Nurgali *et al*., 2018; Schirrmacher, 2019). Additionally, some chemotherapeutic compounds such as doxorubicin confer lifetime dose limits of not exceed 550 mg/m2, which means that a patient can only receive treatment with cumulative lifetime dose of less than the limit or else will encountered with high risk of cardiotoxicity (Cai *et al*., 2019). Arising from these issues, research focus has since shifted into the utilization of nanotechnology to increase the efficacy and at the same time upsurge the safety of the treatment. The encapsulation of therapeutics by nanoparticles can potentially increase the bioavailability and cellular uptake, thereby can reduce the total dosage of therapeutics to be consumed by patients (Wolfram *et al*., 2015).

There are many different kinds of nanoparticle systems being formulated by researchers such as carbon nanotube, solid lipid nanoparticle, chitosan nanoparticle and metal nanoparticle. These nano-scale systems generally are 10-100 nm in diameter and able to encapsulate with various cargos attributed to their own properties. The encapsulation of cargos can protect them from random enzymatic degradation and increase the bioavailability, in turn increase the cellular uptake by increasing cellular accumulation (Behzadi *et al*., 2017; Foroozandeh & Aziz, 2018; Z. Li *et al*., 2015). Furthermore, recent studies have reported that nanoparticle systems gain the advantage of enhanced permeability and retention effect (EPR) of tumor cells (Kang *et al*., 2020; J. Park *et al*., 2019). This means they can significantly increase the cellular accumulation at targeted tumor by crossing the blood vessels and tumor vasculature system present in the body (Golombek *et al*., 2018; Kalyane *et al*., 2019).

Chitosan nanoparticle system has emerged as a promising nanoparticle system for the delivery of various kinds of cargos including therapeutic drugs, natural compounds and genes. Chitosan-based nanoparticles possessed positively surface charge and mucoadhesive characteristics which can promote the penetration across the epithelial barriers to facilitate the delivery of cargos

(Mohammed *et al*., 2017). Besides, the ease of preparation through ionic gelation method with cross-linker such as sodium tripolyphosphate (TPP) with optimized parameter capable to synthesize monodispersed chitosan nanoparticles (CNPs). The CNP system is enriched with free amine groups due to N-acetyl glucosamine of chains of chitosan polymer, which provides a conjugation point for compounds and therapeutic genes that are able to conjugate with the amine groups (Sonia & Sharma, 2011). Additionally, the biodegrable and biocompatible properties of CNP have also eliminated that potential problem of accumulation of CNP after consumption (Pang *et al*., 2017). Therefore, these advantages of CNP have suggested the choice of CNP as a promising delivery system as compared with orther nanoparticle systems. Nevertheless, this system is not an ideal route for encapsulation of poorly water soluble compounds such as silibinin (SLB) due to its hydrophobicity and non-charged surface (Biedermann *et al*., 2014). Although other nanoparticle system, for example solid lipid nanoparticle system is also one of the well-known nano-system for poorly soluble compounds, chitosan nanoparticle derivative system with positive surface charge is preferred. It is because positively charged nanoparticles appeared to have greater cellular uptake efficacy than negatively charged nanoparticles (Jeon *et al*., 2018).

Silibinin (SLB) is an active natural compound extracted from Silybum marianum, commonly known as milk thistle plant. SLB exists as polyphenolic flavonolignan with prominent hepatoprotective and antioxidant activity (Surai, 2015). This natural compound has gained more attentions from researchers since substantial evidences from past and current study have demonstrated its clinical use worldwide (Ferenci, 2016; Tamayo & Diamond, 2007). Furthermore, the utilization of natural compounds can in turn eliminate or minimize the undesirable side effects of synthetic drugs (Mitra & Dash, 2018). However, the utilization of SLB was hindered by constrains of low water solubility and poor cell accumulation (Di Costanzo & Angelico, 2019). The drawbacks of traditional CNP system of low encapsulation and therapeutic efficacy of low water solubility compounds have led to the formulation of hydrophobically-modified chitosan nanoparticle (pCNP) system through the conjugation of NHS-palmitic acid by using the N-hydroxysuccinimide ester group (-NHS) of palmitic acid. The conjugation of the NHS-palmitic acid introduced the hydrophobic moieties to the chitosan polymer and it was hypothesize will be more competent in encapsulation of SLB through the hydrophobic-hydrophobic interactions between SLB and NHS- groups. Palmitic acid is one of the most common saturated fatty acid that can be found naturally in various diet, and existed as an essential component of cell membranes, secretory and transport lipids (German, 2011). In addition, it was reported that the homeostasis of palmitic acid was regulated by endogenous biosynthesis via de novo lipogenesis to prevent its accumulation after consumption (Carta *et al*., 2017). Thus, the employment of palmitic acid as hydrophobic moiety in this study can potentially avoid undesirable allergic problem and minimum health related concerns.

## 1.2 Statement of research problem

The cancer disease remains the major cause of mortality globally since many decades ago. Chemotherapeutic agents have been arisen as most common treatment for cancer disease. However, the employment of natural occurring compounds such as phenolic compounds have gained more attention from researchers due to the undesirable side effects following chemotherapy treatment (Mitra & Dash, 2018; Zhang *et al*., 2018). Phenolic compounds have low water solubility and low accumulation in targeted cells, leading to inefficient delivery routes in treatments. In order to increase the loading, efficacy and release of such compounds, higher dosing needs to be administered leading to non-specific destruction of normal cells and multidrug resistances upon consumption or injection into the human body.

The delivery of therapeutics need the presence of delivery vehicle to protect themselves from enzymatic degradation and enhance the efficacy. Although various delivery vectors including several nanoparticle systems have been synthesized and utilized for the delivery of different therapeutics, the delivery of poorly water-soluble phenolic compounds however required additional attention due to their poor bioavailability and low cellular accumulation. A holistic delivery system for poorly water-soluble compounds in cancer treatment was able to conjugate with the compounds and possessed controlled release properties after administered by patients. Thus, this study is focused on developing a nano-delivery system where can form hydrophobic linkage with hydrophobic compounds to increase the encapsulation efficiency and possessed controlled release properties after administration.

## 1.3 Research statement of study

Conventional cancer treatments such as chemotherapy and radiotherapy have occasioning a series of undesirable side effects such as cardiotoxicity, nausea, and hair loss in patients (Nurgali *et al*., 2018). Therefore, the utilization of natural therapeutic alternatives such as plant extracts, herbs and phenolic compounds as anticancer agents have gained attention from researchers to reduce the dosing of therapeutics through nanoparticle encapsulation (Kooti *et al*., 2017). Nonetheless, the therapeutic efficacy of some of these natural alternatives was restricted by some limitations such as low water solubility which leads to poor bioavailability and low cellular accumulation. This study is deliberate to synthesize a novel hydrophobically-modified chitosan nanoparticle (pCNP) system to increase the encapsulation efficiency of poorly water soluble compounds and subsequently enhance the therapeutic efficacy and controlled release properties. The pCNP system was synthesized through the conjugation of NHS-ester group of NHS-palmitic acid to the amine group of chitosan. Consequently, the NHS-ester groups in chitosan act as hydrophobic moieties associated with poorly soluble compound through hydrophobic-hydrophobic interactions. This pCNP system could serve as potential delivery vehicle for various poorly soluble compounds to aid in medical and pharmaceutical fields.

## 1.4 Hypothesis of study

Modification to the structure of CNP, which includes a hydrophobic moiety such as palmitic acid will increase the encapsulation efficiency, compound-nanoparticle interaction, and confer sustained release properties of silibinin from nanoparticles.

## 1.5 Objectives

The general objective of the study was to develop a hydrophobically-modified chitosan nanoparticle system to increase the loading, controlled release and efficacy of poorly-soluble phenolic compound, silibinin (SLB) for increased anti-cancer activities.
The specific objectives is described as follow:

1. To perform hydrophobic modification of conventionally synthesized chitosan nanoparticles using NHS- conjugation with palmitic acid;

2. To characterize the hydrophobically modified-chitosan nanoparticles using various physico-chemical characterization analysis;

3. To evaluate the cytotoxicity efficacy of SLB, CNP-SLB, and pCNP-SLB in various human cancer cell lines; and

4. To analyze the enhanced therapeutic efficacy and sustained release properties of pCNP-SLB in apoptotic cell death and effect in cell cycle.

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The author was born in Hospital Teluk Intan, Perak Darul Ridzuan in 1991. Due to the hardship of life, he has to follow his parent to move to several places before settled down in a small town, Langkap. He went through his primary education in S.J.K (C) Wah Keow, Langkap, and later attended secondary education in S.M.K Dato' Sagor, Langkap. After SPM, he continued his study in S.M.K Horley Methodist Teluk Intan to pursue his pre-university (STPM) course. Fortunately, he was then offered by Universiti Putra Malaysia for his Bachelor degree for 4 years. He received his Bachelor of Science (Honours) in Cell and Molecular Biology course in 2015. Next, after worked as research assistance for about 4 months, he continued his post-graduate study journey in Master of Science. In January 2018, he has successfully converted his student status and started his PhD journey after a series of conversion examination at Faculty of Biotechnology and Biomolecular Sciences.

## LIST OF PUBLICATIONS

**Paper publications:**

Kuen, C. Y., Fakurazi, S., Othman, S., & Masarudin, M. (2017). Increased Loading, Efficacy and Sustained Release of Silibinin, a Poorly Soluble Drug Using Hydrophobically-Modified Chitosan Nanoparticles for Enhanced Delivery of Anticancer Drug Delivery Systems. *Nanomaterials*, 7(11), 379.

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Kuen, C. Y., Galen, T., Fakurazi, S., Othman, S. S., & Masarudin, M. J. (2020). Increased Cytotoxic Efficacy of Protocatechuic Acid in A549 Human Lung Cancer Delivered via Hydrophobically Modified-Chitosan Nanoparticles As an Anticancer Modality. *Polymers*, 12(9), 1951

Kuen, C. Y., Fakurazi, S., Othman, S., & Masarudin, M. Enhanced Therapeutic Efficacy of Silibinin via a Nano-Mediated pCNP Delivery System towards A549 Human Lung Cancer Cells. (in the process of submission to International Journal of Nanomedicine)

**Conferences and symposium:**

1. Participant in International Congress of the Malaysian Society for Microbiology (ICMSM) 2017, Hotel Bangi Putrajaya.

2. Kuen, C., Fakurazi, S., Othman, S., & Masarudin, M. (2019). Hydrophobic modification of chitosan nanoparticles for increased therapeutic delivery, sustained release, and anticancer properties of silibinin against A549 human lung cancer cell line. Oral speaker at the 3rd International Conference on Molecular Biology & Biotech (ICMBB) 2019. 24-25 April 2019. UCSI University, Kuala Lumpur.

3. Kuen, C., Fakurazi, S., Othman, S., & Masarudin, M. (2019). The enhanced therapeutic efficacy of silibinin encapsulated hydrophobically-modified chitosan nanoparticles in A549 human lung cancer cell linel. Oral presenter at the Pure and Applied Biology Colloqium (PABC) 2019. 30th October 2019. Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam (Best Oral Presenter)

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