UNIVERSITI PUTRA MALAYSIA

FORMULATION, CHARACTERIZATION AND CYTOTOXICITY EVALUATION OF OROTIC ACID AND MAGNESIUM OROTATE LOADED INTO GUM ARABIC AND CHITOSAN NANOPARTICLES FOR DRUG DELIVERY

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By

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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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Controlled drug release has been used to improve the bioavailability properties of various drugs. These systems enable better regulation of drugs administered for treatments and reduce their side effects in therapeutic levels with minimum concentrations. In this study, gum arabic (GA) and chitosan (CS) nanoparticles were used as nanocarriers to encapsulate orotic acid (OA) and magnesium orotate (MgOr) due to their attractive physicochemical properties which can improve targeted drug delivery. Therefore, the main objectives of the current study were to develop a nanomaterial-based carrier as a novel drug delivery system of OA and MgOr by using gum arabic nanoparticles (GANPs) and chitosan nanoparticles (CSNPs) for enhanced delivery efficiency.

Then, the antioxidant and *in vitro* antihypertensive properties of the nanoparticles (NPs) were assessed. Comparisons were made between active compounds, respective polymers and synthesised nanopartilces (NPs) in terms of their antioxidant, antihypertensive and cytotoxicity properties. The resulting four NPs, namely MgOrGANPs, MgOrCSNPs, OAGANPs and OACSNPs, were prepared using the freeze-drying technique. The physicochemical characteristics of NPs, specifically the functional groups, crystallinity, thermal behaviour, surface morphology, and drug loading percentage, were examined using Fourier-transform infrared spectroscopy (FTIR), X-ray diffractometry (XRD), differential scanning calorimetry (DSC), and transmission electron microscopy (TEM). Furthermore, the antioxidant potential activities of orotic acid nanoparticles (OANPs) and magnesium orotate nanoparticles (MgOrNPs) were assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), and β-carotene bleaching assays. Apart from that, the antihypertensive activity was performed using angiotensin-converting enzyme (ACE). In addition, HepG2 (human liver cancer cell lines), MCF7 (human breast cancer cell lines), HT29 (human

colon cancer cell line), MCF10A (normal breast cell lines), ARPE-19 (human retinal epithelial cell line), and 3T3 (mouse fibroblast cell line) were treated with NPs for cytotoxicity evaluation. Meanwhile, The FTIR, XRD and DSC analysis confirmed the encapsulation of OA and MgOr into GA/CSNPs. The initial burst of drugs was improved with polymer coating agents, resulting in their controlled release of drugs from their nanoparticles. On the other hand, the preliminary in vitro cytotoxicity tests suggested that OANPs and MgOrNPs were not acutely toxic and significantly inhibit the growth of cancer cells. Thus, the findings demonstrated that polymer coating significantly improved the antioxidant, antihypertensive and cytotoxicity properties of drug-loaded nanoparticles compared to the uncoated ones. In conclusion, the desirable characteristicsof of the OANPs and MgOrNPs that were developed in this study have the potential as drug nanocarriers to deliver poorly water-soluble drugs OA and MgOr.

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

PENILAIAN PERUMUSAN, PENCIRIAN DAN KESITOTOKSIKAN ASID OROTIK DAN MAGNESIUM OROTAT YANG DIBEBANKAN KE NANOPARTIKEL GAM ARAB DAN KITOSAN UNTUK PENGHANTARAN UBATAN

Oleh

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Pelepasan ubat yang terkawal digunakan untuk meningkatkan sifat biokeperolehan pelbagai ubat. Sistem ini membolehkan pengawalan ubat yang lebih baik yang diberikan untuk rawatan dan mengurangkan kesan sampingannya pada tahap terapi pada tahap kepekatan minimum. Dalam kajian ini, nanopartikel gam arab (GA) dan kitosan (CS) digunakan sebagai pembawa nano untuk merangkum asid orotik (OA) dan magnesium orotat (MgOr) kerana sifat fizikokimia yang wujud dapat meningkatkan penghantaran ubat ke sasaran. Objektif utama kajian ini adalah untuk membangunkan pembawa berasaskan bahan nano sebagai sistem penyampaian ubat baru OA dan magnesium orotat (MgOr) dengan menggunakan nanopartikel gam arab (GANPs) dan nanopartikel kitosan (CSNPs) untuk meningkatkan kecekapan penghantaran. Kemudian, sifat antioksidan dan nanopartikel antihipertensi in vitro (NPs) dinilai. Perbandingan dilakukan di antara sebatian aktif, polimer masing-masing dan NP yang disintesis daripada segi sifat antioksidan, antihipertensi dan kesitotoksikan. Empat NP yang dihasilkan, iaitu MgOrGANPs, MgOrCSNPs, OAGANPs dan OACSNPs dimendakkan menggunakan teknik pengeringan sejuk beku. Ciri fizikokimia NP, khususnya kumpulan berfungsi, kekristalan, perlakuan terma, morfologi permukaan, dan peratusan pemuatan ubat, diperiksa menggunakan spektroskopi inframerah transformasi Fourier (FTIR), difraktometer sinar-X (XRD), kalorimetri pengimbasan pembezaan (DSC) dan mikroskop elektron penghantaran (TEM). Tambahan pula, aktiviti potensi antioksidan nanopartikel asid orotik (OANPs) dan nanopartikel magnesium orotat (MgOrNPs) dinilai menggunakan 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitrik oksida (NO) dan asai peluntur β-karotena). Selain itu, aktiviti antihipertensi dilakukan menggunakan enzim pengubah angiotensin (ACE). Tambahan pula, HepG2 (titisan sel barisan sel barah hati manusia), MCF7 (titisan sel barah payudara manusia) HT29 (titisan sel barah kolon manusia), MCF10A (titisan sel payudara normal), ARPE-19 (titisan sel epitel retina manusia) dan 3T3(titisan sel fibroblas tikus) dirawat dengan NP untuk penilaian kesitotoksikan. Sementara itu,

analisis FTIR, XRD dan DSC mengesahkan pengkapsulan OA dan MgOr ke GA / CSNPs. Deretan awal ubat diperbaiki dengan agen pelapisan polimer, mengakibatkan pelepasan ubat terkawal dari nanopartikel. Sebaliknya, ujian kesitotoksikan in vitro awal menunjukkan bahawa OANPs dan MgOrNPs tidak beracun secara akut dan secara signifikan menghalang pertumbuhan sel barah. Oleh itu, dapatan menunjukkan bahawa lapisan polimer dengan ketara meningkatkan sifat antioksidan, antihipertensi dan sitotoksisiti nanopartikel yang dimuatkan ubatan berbanding yang tidak dilapisi. Kesimpulannya, ciri-ciri OANP dan MgOrNP yang diinginkan dalam kajian ini berpotensi sebagai pembawa nano ubat untuk menyampaikan ubat larut air OA dan MgOr.

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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 the degree of Doctor of Philosophy.The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

OA	Orotic acid
MgOr	Magnesium orotate
CS	Chitosan
GA	Gum arabic
OANPs	Orotic acid nanoparticles
MgOrNPs	Magnesium orotate nanoparticles
OAGANPs	Orotic acid-loaded gum arabic nanoparticles
OACSNPs	Orotic acid-loaded chitosan nanoparticles
MgOrGANPs	Magnesium orotate -loaded gum arabic nanoparticles
MgOrCSNPs	Magnesium orotate -loaded chitosan nanoparticles
EE	Encapsulation efficiency
DPPH	1,1-diphenyl-2-picrylhydrazyl)DPPH(,
NO	Nitric oxide
MTT	-)4,5-dimethylthiazol-2-yl(-2,5-diphenyltetrazolium bromide
3T3	Normal fibroblast
ARPE-19	Normal retinal pigmented epithelial
HepG2	Hepatocellular carcinoma
MCF7	Michigan Cancer Foundation 7
HT29	Human colon adenocarcinoma
MCF-10A	Michigan Cancer Foundation 10A
PLGA	(poly(lactic-co-glycolic acid)
DMEM	Dulbecco's modified Eagle's medium
RAW 264.7	Murine monocytic macrophage
LPS	Lipopolysacchairde

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The controlled-release formulation at a specific site within optimum time and protection of bioactive agents has been made possible via the encapsulation technique. Nanoencapsulation is considered the most effective technology to entrap various bioactive agents. It is both feasible and advantageous for efficient absorption by different human cells and targeted site-specific delivery (Behnaz et al., 2019). Moreover, it allows the formulation of many pharmaceutical products, protecting; reforming and improving their bioactivity in the body (Muthukrishnan et al., 2019). Nanoencapsualtion not only results in enhanced drug formulation but also improved oral or parenteral delivery systems. At present, numerous encapsulated products are marketed mainly as pharmaceutical products (Puneet & Subramony, 2018).

Polymeric nanoparticulate is considered a drug delivery systems that can be administered in various forms such as nanospheres, nanocapsules and nanoparticles (Erdoğar et al., 2018). Despite the invention of nanotechnology and knowledge advancement in pharmaceutical chemistry, molecular biology, and bioscience, the significant changes in the approaches and methodologies of the drug delivery systems have introduced new challenges.

Now, there is a need for new nanomedicine devices and drug formulations to suit the requirements of molecular drug delivery systems (Makkizadeh, 2018). Nanoparticles offer a solution to this predicament with their ability to improve hydrophobic properties of various compounds and deliver them to tissues and specific target sites for cancer treatment (Chen et al., 2018). Nanoparticles, which often measure between 1 nm and 10 nm, have gained prominence in drug delivery systems due to their physicochemical properties and enhanced performance (Jeevanandam et al., 2018).The prepared nanoparticles target diseased tissues (e.g., cancer treatment) to protect healthy human body cells and perform preliminary diagnostics of diseases (Richel et al., 2019). Nanomedicine is beneficial for numerous medical applications due to its unique characteristics, such as surface ratio, size distribution, quantum properties, and adsorption capacity of the biocompounds (Daniel et al., 2018).

Drug delivery refers to administering or using pharmaceutical compounds to achieve potential therapeutic targets in animals or humans. For this reason, numerous drug delivery release systems have been improved and explored for oral, pulmonary, and nasal delivery, including nanoparticles, liposomes, gels, and proliposomes. In most cases, drug delivery systems that involve nanoparticles consist of biodegradable polymers, which display high efficiency to meet the requirements of these delivery

systems (e.g., stability, biocompatibility, and site-specific targets) (Kyoung et al., 2018). Most of these drugs are characterised by their capacity to release bioactive agents at specific targets within the expected time.

Many studies have suggested the application of biocompatible and biodegradable functional forms and techniques to improve these properties.

Furthermore, these techniques are used to control toxicity, concentration of bioactive ingredients, and drug loading efficiency (Xue et al., 2019). The importance of drug bioavailability in terms of nanoencapsulation efficiency has been widely acknowledged.

The technique is also used for medical purposes, such as after the oral administration for improved therapeutic activity and bioavailability of drugs (Qilong et al., 2018). Its capability to enhance the hydrophobic properties of drugs has propelled the use of these systems to deliver drugs to the target sites. Nanoparticle systems are prepared to overcome the limitations of cancer therapy in conventional treatments and diagnostics. The uses of biodegradable polymers as coating materials, such as CS and GA, are highly significant for researchers and patients as well as in the field of nanomedicine and nanotechnology, as these polymers can be loaded with potential bioactive and therapeutic substances with operational stability, such as proteins, vitamins, and antioxidants (Juan et al., 2018; Ida et al., 2018; Manan et al., 2017).

Gum Arabic (GA) is essential for a wide range of nanoparticles in the drug delivery system given its capabilities to enhance colloidal stability and offer relevant functional groups for the coupling of bioactive agents (Sarika et al., 2015). GA refers to a common polysaccharide from Acacia species, which is used in numerous biomedical applications. Besides that, its encapsulation properties and unique emulsification evaluated the toxicology assessements of drugs (Zulaikha et al., 2018). GA is a polysaccharide-coating material with antioxidant and antihypertensive properties. It has been reported that this material inhibits ethylene fabrication and prevents dehydration process. Among its many properties, the strong antioxidant property of the natural polysaccharide like chitosan is the most widely documented (El-Batal et al., 2018).

Natural polymers, such as chitosan (CS), are usually biocompatible, biodegradable, and inexpensive. CS, which is one of the natural biodegradable polymer groups, is extensively used for the microencapsulation of drugs like isoniazid, propranolol, and aspirin. This natural polysaccharide benefits many pharmaceutical applications, such as oral and parenteral delivery of drugs. It is important for a wide range of scientific and industrial processes to recognise the applications of CSNPs loaded drugs in the pharmaceutical field. Recently, this issue was the objective of many research papers in the literature. CS can also be combined with other polymers for the encapsulation of many drugs in order to achieve targeted performance delivery.

The recent advancement in the nanoencapsulation methods has facilitated studies on the use of CS to load drugs. CS is a natural, biodegradable, and linear polysaccharide that consists of distributed (deacetylated units) that enable slow/controlled drug release, which reduces toxicity and enhances stability and solubility of drugs (Kabo et al., 2018).

1.2 Problem statement

There are various limitations in the current techniques used to administer conventional drugs via tablets or liquids, such as low solubility and limited drug efficacy.

Orotic acid (OA) is a pyrimidine carboxylic acid that serves as an intermediate in the synthesis of pyrimidine (Figure 1.1).

Most studies tend to focus on the antitumour and anti-inflammatory activities of OA. Orotic acid is manufactured in the human body from dihydroorotate dehydrogenase enzyme (Kostova et al., 2015).

The presence of non-covalent recognition sites, van der Waals forces, hydrogen bonds and carboxylate group in orotic acid improve ligand binding properties and accumulation into a higher-dimensional product based on multifunctional and supramolecular frameworks (Siddiqui et al., 2016).

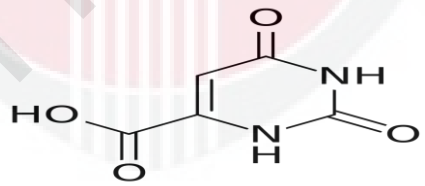

Figure 1.1 : Structure of orotic acid
(Source : Kostova et al., 2015)

Magnesium orotate (MgOr) aids the development of various biological components as an anticancer and antihypertensive agent (Hacht & Taaya, 2006) (Figure 1.2).

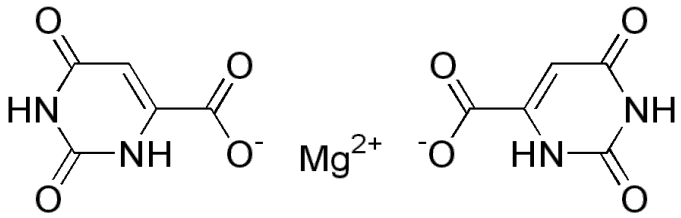

Figure 1.2 : Magnesium orotate structure
(Source: Matthew et al., 2015)

The conversion and absorption of orotate can be improved in the presence of magnesium through the modulation of uridine metabolism (Matthew et al., 2015). The penetration of OA through cells can be performed via the uracil transporter (Matthew et al., 2015). Moreover, magnesium orotate complex has been used as a therapeutic compound for the treatment of cancer (Kafeel et al., 2016). The potential effects of MgOr and OA are limited due to their poor water solubility. Furthermore, the maximum percentage of drugs administered can be immediately metabolised before reaching the therapeutic targets.

Therefore, polymeric nanoparticle delivery systems with lower drug dosages can improve the solubility, bioavailability and targeting properties of MgOr and OA (Kabo et al., 2018; Juan et al., 2018). The unique properties of nanoparticles (e.g., large surface-to-volume ratio) enhance the therapeutic effectiveness of components with specific shapes and sizes (Narges et al., 2018).

Chitosan is a biodegradable, non-toxic polysaccharide widely used in drug delivery systems due to its ideal surface properties depending on its chemical structure (Ren et al., 2019). Chitosan nanoparticles were used in controlled-release systems to improve the effectiveness of orotic acid therapy (Wafaa et al., 2018) (Figure 1.3).

The intercalation of neutral OA into CS provides a positive charge on its surface, leading to favourable endocytosis of cells and subsequently enhancing the anticancer activity. The repulsive force generated with negative charges of cell walls prohibits the cellular internalisation of various drugs (Alberto et al., 2018). A previous study described the use of CS in improving oral bioavailability and explored the mucoadhesive characteristics of CS (Wang et al., 2018) . It is also used to increase the stability of drugs, enhance tumour targeting, and control the release of hydrophilic compounds (e.g., metformin-coated liposomes of CS and glycerolphosphate) (Sanjurjo et al., 2019)**.** Therefore, the control of the cationic nature of CS is appropriate for maintaining the stability of ionic complexes over a wide range of pH values.

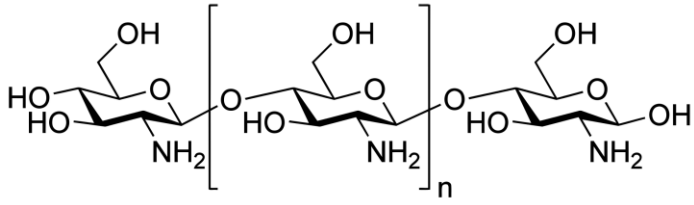

Figure 1.3 : Structure of chitosan
(Source : Islam et al., 2017)

GA or acacia gum is frequently grown in Africa, India, and Australia. It is a natural gum that is extracted from branches and stems of Acacia Senegal (Leguminosae) (Figure 1.4). Its hydrophilic, non-toxic glycoprotein polymer serves as a stabiliser for pharmaceutical and food applications (Elshama, 2018). Furthermore, it is an amphiphilic polysaccharide with good stability at high temperature and high ionic strength environments
(Ren et al., 2019).

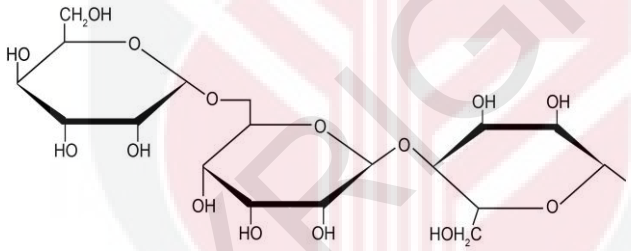

Figure 1.4 : Gum Arabic structure
(Source : Sarika et al., 2015)

Its antioxidant activity, low viscosity at high temperature, binding properties, and non-toxic glycoprotein polymer makes it a good stabiliser in the pharmaceutical and food industry. Moreover, the presence of galactose groups in GA improves its anticancer activity (Sarika et al., 2015). The highly branched molecular structure of GA enhances colloidal stability, *in vitro* stability and induces the steric repulsion properties of NPs, whereas the carboxyl groups are linked to biocompounds (Guowen et al., 2019; Arora et al., 2016; Andreea et al., 2018).

The synthesised NPs can cross cell barriers through the enhanced penetration and retention effect with minimal harm to the normal cells. Therefore, GANPs and CSNPs were selected as nanocarriers for the effective delivery of OA and MgOr in this study.

1.3 Scope of study

The current study is carried out to determine and develop the physicochemical properties of GANPs and CSNPs for drug delivery of OA and MgOr. Release profiles and drug loading of MgOrNPs and OANPs were assessed at pH 4.8 and pH 7.4. The cytotoxicity properties of drug-loaded nanopartilces were performed againts normal cell lines (3T3, ARPE-19, and MCF10-A) and human cancer cell lines (HepG2, MCF7, and HT29). Additionally, the antioxidant and antihypertensive properties of NPs compared to its active compounds, OA and MgOr, were determined using DPPH, nitric oxide, β-carotene and angiotensin-converting enzyme (ACE) assays.

1.4 Hypothesis of the study

As one of the most important components of drugs and compounds developments, nanoencapsulation is disseminated in several industrial fields and offers various advantages as a drug delivery system. With these unique advantages of NPs, the use of GA/CSNPs as a promising drug delivery system for advanced therapeutic treatment is evident. GANPs and CSNPs can be loaded with OA and MgOr via encapsulation process for drug delivery. The developed MgOrGANPs, MgOrCSNPs, OAGANPs and OACSNPs drug-loaded nanoparticles indicated controlled-release properties with improved efficiency of the *in vitro* delivery of OANPs and MgOrNPs compared with active compounds and coating agents alone.

Due to their non-toxic, biodegradable and bioavailability properties, the drug-loaded nanoparticles with lower therapeutic drugs dosages protect healthy cells *in vitro*. Therefore, they can improve the antioxidant, antihypertensive properties of drugs by inhibiting the growth of cancer cell lines *in vitro*.

1.5 Objectives

The study aims to develop drug-loaded, polymeric GANPs and CSNPs for effective drug delivery. The specific objectives are as follows:

1. To characterize and determine the properties of the nanopartilces, MgOrGANPs, MgOrCSNPs, OAGANPs and OACSNPs.
2. To investigate the cytotoxicity of MgOrGANPs, MgOrCSNPs, OAGANPs, and OACSNPs in normal cell lines (*i.e* .3T3, ARPE-19, and MCF10-A) and human cancer cell lines (*i.e*. HepG2, MCF7, and HT29) and examine the antioxidant and antihypertensive properties of NPs in comparison with its active compounds, OA and MgOr, respectively.
3. To compare the effects of coating agents and their active compounds with synthesised NPs in terms of cytotoxicity, antioxidant, and antihypertensive properties.

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