IN VITRO ASSESSMENT OF ANTIMICROBIAL PROPERTIES OF MALAYSIA MANGROVE PLANT (*RHIZOPHORA APICULATA)* FOR THE APPLICATION IN BONE REPLACEMENT MATERIALS.

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UNIVERSITI SAINS MALAYSIA
2012

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by

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Thesis submitted in partial fulfillment
of the requirement for the degree
of Master of Science

August 2012

ACKNOWLEDGEMENT

In the name of Allah the most beneficent and the most merciful, all praise and thanks are due to Allah the creator and sustainer of the worlds and His Messenger Muhammad S.A.W for his bond of love.

I would like to address my deepest wholehearted gratitude to my supervisor, Associate Prof. Dr. Sharif Hussein Sharif Zein, for his continuing support and mentorship in completing this project within limited time frame. I would like to thank him for his enthusiasm and understanding that was shown throughout the period. My accomplishment of this research is direct reflection of high quality supervision from my supervisors.

In addition, I would like to express my gratitude to administrative staff of School of Chemical Engineering, University Sains Malaysia especially our respected dean, Prof. Azlina Harun@ Kamaruddin, deputy dean, office staff and technicians for giving me full support throughout my research work.

Special thanks to my beloved friend especially to Siti Maisurah bt. Zakaria and Fatemeh Gholomi for their full support given to me during my study. I might not able to achieve what I want to be without the support from my friends. My appreciation also goes to them who had stick with me through hardship and joy.

Finally, I would like to take this opportunity to thanks my parents for support, eternal dedication and devotion which had shaped and inspired my life.

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ATM	Antimicrobial agent
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
CPCs	Calcium phosphate ceramics
DI	Deionized water
FTIR	Fourier transform infrared spectrometer
HA	Hydroxyapatite
MBC	Minimum bactericidal concentration
MIC	Minimum inhibition concentration
MWCNTs	Multi-walled carbon nanotubes
KBr	Potassium Bromide
PMMA	Polymethylmethacrylate
PJI	Prosthetic joint infections
SEM	Scanning electron microscope
SBF	Simulated body fluid
TTCP	Tetracalcium phosphate
TEM	Transmission electron microscope
TPC	Tricalcium phosphate

LIST OF SYMBOLS

°C	Degree celcius
wt%	Weight percentage
v/v	Volume/volume
wt/vol	Weight/volume
MPa	Mega pascal
t	Incubation time
M	Mass transferred with respect to time
S	Solid particle of instantaneous surface
Cs-Ct	Concentration driving force
D	D is the diffusion coefficient
γ	Solution volume
h	Diffusion layer thickness
Q_t	Cumulative amount of drug dissolved in time t
Q_0	Initial amount of drug in the solution
K_0	Zero order release
k	First order rate constant
k	Rate constants
K_H	Higuchi dissolution constant
K_{HC}	Rate constant for Hixson-Crowell rate equation
Mt / M∞	Fraction of drug released at time
R_{HA}	Radius of the HA particle
g	Function of the additive concentration
X_1	Weight of the dry powdered barks before extraction,
X_2	Weight of the dry bark after extraction.
g	Gram
µL	Micro mile
kV	Kilo watt
Nm	Nanometer

PENILAIAN LUAR BADAN OLEH AKTIVITI AGEN ANTI BAKTERIA DARIPADA POKOK BAKAU, MALAYSIA (*Rhizophora Apiculata*) UNTUK APLIKASI PENGANTIAN TULANG.

ABSTRAK

Objektif kajian ini ialah untuk menilai aktiviti luar badan oleh agen anti bakteria (ATM) daripada kulit kayu pokok bakau, Malaysia (*Rhizophora Apiculata)* yang digunakan untuk mencegah aktiviti bakteria dalam badan manusia dan diaplikasikan sebagai bahan pengantian tulang. Ekstrak tanin daripada kulit *Rhizophora Apiculata* menunjukkan keputusan dan prestasi yang baik sebagai agen anti bakteria. Tambahan pula, komposit hidroksiapatit (HA) juga meningkatkan tahap anti bakteria dan berpotensi tinggi dalam aplikasi pengantian tulang. Ekstrak tanin mengunakan "acetone" dengan nisbah 1:5, menghasikan antara 33-35% agen anti bakteria daripada tanin. Komposit HA/ATM dengan peratusan ATM antara 10-50% digunakan dalam kajian ini. Kaedah anti bacteria dilakukan dengan menggunakan tiga spesis bakteria badan manusia iaitu *Staplococcus aeurus, Staplococcus epidermis and Pseudomonas aeruginosa.* Kaedah resapan bakteria menunjukkan komposit HA/ATM telah berjaya merencatkan ketiga-tiga bakteria ini. Oleh itu, purata zon perencat daripada aktiviti anti bakteria meningkat apabila peratus ATM dalam komposit HA/ATM meningkat daripada 10 kepada 50%. Kekuatan mekanikal komposit HA/ATM juga dapat dinilai dengan kekuatan mekanikal yang tertinggi daripada komposit dengan 40% ATM (6.65 ± 1 MPa) diikuti oleh 30% ATM (5.61 ± 1 MPa) dan 50% ATM (5.46 ± 1 MPa). Keputusan ini juga menunjukkan campuran daripada ATM kepada HA boleh menyumbangkan kepada peningkatan kekuatan mampatan untuk HA. Selain itu,

morfologi dan struktur HA/ATM juga dianalisis dengan menggunakan SEM. Kajian mendapati kehadiran ATM sememangnya memberi kesan kepada morfologi dan struktur dalam kristal HA. Model matematik dalam pembebasan kinetik juga dikembangkan berdasarkan sistem tahap pembebasan. Berdasarkan keputusan kinetic pembebasan, model "Higuchi" adalah model yang terbaik dalam model kinetik berdasarkan daripada keputusan R^2 paling tinggi dan menunjukkan kadar pembebasan ATM daripada HA adalah punca kuasa dua dengan masa. Selain itu, tindak balas tertib juga terlibat dalam kinetik pertumbuhan HA dianalisis melalui kaedah intergrasi. Tindak balas berdasarkan "Zero-order" adalah model terbaik dengan menunjukkan kadar tindak balas untuk kinetik pertumbuhan HA adalah berkadaran dengan masa tindak balas.

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ABSTRACT

The aim of this study is to conduct in vitro assessment of antimicrobial (ATM) properties of Malaysia mangrove plants (*Rhizophora Apiculata)* barks against human pathogen bacteria for bone replacement application. Tannin extracted from *Rhizophora Apiculata* barks has shown a very good and significant performance as an ATM agent. In addition, development of hydroxyapatite (HA) composites with enhanced antimicrobial property are potentially attractive in of bone replacement applications. The extraction of tannin using acetone in ratio 1:5, gave practically about 33-35% of raw tannin as an ATM agent. HA/ATM composites with the ATM percentage in the range of 10-50% were used throughout the studies. Antimicrobial activity test was performed by using three human pathogen bacteria which are *Staplococcus aeurus, Staplococcus epidermis and Pseudomonas aeruginosa.* The disc diffusion test showed the HA/ATM agent was successfully susceptible against these three bacteria. In addition, the mean inhibition zones of antimicrobial activity were increased with the increasing of the ATM percentage in HA/ATM composites from 10 to 50%. Mechanical properties of the HA/ATM composite were also evaluated and the highest mechanical strength was given by HA/ATM composite with 40% ATM (6.65 ± 1 MPa) followed by 30% ATM (5.61 ± 1 MPa) and 50% ATM (5.46 ± 1 MPa). The results showed that the concomitant admixture of ATM to pure HA considerably increased the compressive strength of HA. Besides that, characterization on the morphology and structure of

HA/ATM was done by using SEM. The presence of ATM was found to have significant effects in influencing the morphology of HA crystals. The mathematical model of ATM kinetic release was developed based on the drug release system. By refering to the kinetic release result, it has shown the Higuchi's model is the best-fit model kinetic regarding to the highest R^2 value and indicated that the ATM release from HA matrix as a square root of time dependent process based on diffusion process. Moreover, the reaction order for the reaction involved in HA kinetics growth was estimated through the integral method. Zero-order reaction is the best-fit model which showed that the reaction rate of kinetic growth for HA formation is proportional to the incubation time.

CHAPTER ONE

INTRODUCTION

This chapter provides the detail introduction for overall view of this project. An overview on bone biomaterials, importance of antimicrobial agents in bone grafting and mangrove plants as natural source of antimicrobial agent in the application of bone replacement materials are included in this chapter. It is also included with the problem statement, scope of study, objectives and thesis organization throughout this project.

1.1 Biomaterials for Bone Replacement

Bone replacement is a common but complicated clinical problem in orthopaedic surgery. Every year, millions of people suffer from bone defects arising from trauma, tumor or bone diseases, and inevitably in extreme cases, resulting in death due to insufficient ideal bone substitute (Murugan & Ramakrishna, 2005). Based on the statistic, more than 2.2 million bone grafting procedures are performed in worldwide (Murugan & Ramakrishna, 2005). Furthermore, there are so much effort has been invested through development of biomaterials for the repair or replacement of hard tissues. Besides, the specific consideration for bone replacement materials is the biomechanical nature as a general consideration for biocompatibility. In fact, biomaterials should also possess a good mechanical properties which necessary for a proper performance in their function.

Bone tissue engineering seeks to develop the strategies to heal bone loss due to disease without any drawbacks and impediments on existed clinical autografting and allografting treatments (Langer et al., 1993; Mistry et al., 2005). In previous decades, common bone substitution materials are traditional bone substitutes such as autografts, allografts and xenografts. But in this year, the conventional bone substitutes bear the risk of infection and immune responses which may cause other health problems thus affecting the quality of the substitute. As an alternative, there are various synthetic materials, composites or bioceramics that can be used to solve the problem (Tadic & Beckmann, 2004; Langer et al., 1993; Murugan & Ramakrishna, 2005). However, none of these materials provides a perfect solution for bone healing because there are always remain the questions about mechanical stability, long term in-vivo biocompatibility and biodegradability (Tadic & Beckmann, 2004).

One of the most promising groups of synthetic bone replacements is calcium phosphate ceramics (CPCs). In addition, CPCs contains of hydroxyapatite (HA), tricalcium phosphate (TPC) and tetracalcium phosphate (TTCP), alumina, zirconia, silica based glasses or bioactive glasses and pyrolitic carbons, as a generally terms as biomaterials. But, the most frequent used CPCs are HA and TCP (Ohtsuki et al., 2009; Rabiee, 2011). The main reason for the development of CPCs is their similarities to bone mineral and in some other properties of bone such as biodegradability, bioactivity and osteoconductivity. The perfect bone replacement materials should own high mechanical properties to aid the growing of bone tissue, good biocompatibility, and have high porosity, with a great degree of pore interconnectivity, to enable the bone tissue growth (Hornez et al., 2007a). Otherwise, development of biomaterials for bone substitutes can help in achieving the best possible care for the patient's sake.

HA is one of the most interesting materials for human hard tissue implants as it exists in the human skeletal system and promotes the ability to be bonded chemically with living bone tissues (Tadic et al., 2002). However, its practical clinical applications under load-bearing conditions have been limited due to the natural brittleness and unsatisfactory strength of HA (Hu et al. 2004; Peterlik et al. 2006). HA holds the potential to receive particular attention due to its chemical composition which is similar to bone mineral constituent (Chen et al., 2002). HA consists of highly crystalline and osteoconductive material that can be classified as the most stable calcium phosphate at neutral pH and the most prevalent form of calcium phosphate on the market (Mickiwicz, 2001). Otherwise, in the present of today's technologies, the preparation of HA can be achieved by restorative biomaterials with HA to mimic the chemical composition present in the human body (Rodriquez-Clemente & Lopez-Macipe, 1998). Thus, through development of HA, there are various methods have been applied to produce HA such as precipitation, solid-state reaction, sol gel, hydrothermal and wet chemical processes (Mickiwicz, 2001; Tadic et al., 2002).

1.2 Antimicrobial (ATM) Agent

ATM agents can be defined as the chemical compounds that biosynthetically or synthetically produced which either to destroy or inhibit the growth or metabolism of variety of the microscopic or submicroscopic forms of life. On the basis of their primary activity, they are more specifically called as antibacterial, antifungal, antiprotozoal, antiparasitic, or antiviral agents. There are thousands of antimicrobial agents. But, only a small number of them are safely used as the chemotherapeutic agents which effective in controlling the infectious diseases in plants, animals, and humans. A much larger

number are used in almost every phase of human activity and also in agriculture, food preservation, water, skin, and air disinfection (Perl et al., 2002; Wiart et al., 2004; McGraw-Hill Concise Encyclopaedia, 2002).

Infection is one of the most devastating adverse events that commonly related with the joint replacement surgery. For example, deep infection rates in primary hip replacement surgery are around 0.5% to 2% which resulted in a reduced quality of patient life (Whitehouse et al., 2002). Therefore, ATM agents may prevent cement colonization which indicate their potential in preventing bacterial growth and subsequent biofilm formation in addition of protecting the bone fracture.

Nowadays, the infectious of diseases represents a serious public health problem and sometimes remains as the leading cause of the death. Thus, the world represented the ATM agents as the terms for the treatment of infectious disease to public health (Liao et al., 2007). Since the bacteria accumulation surrounding the biomaterials which known to cause the biomaterials centered infection, it is important to develop some kind of biomaterial with enhanced antibacterial activity as well as the biocompatibility properties (Liao et al., 2007). In terms of bone infection, *Staphylococcus aureus* is one of microbial which can caused a major of biofilm infections associated with dwelling medical devices and most prosthetic joint infections (PJI) (Sandiford et al., 2010). New therapies are needed to prevent the initial adhesion of bacteria to biomaterial surfaces as well as increasing the resistances among impregnating of bone cements (Sandiford et al., 2010). Recently, clinical used synthetic ATM coating with bone biomaterial such as silver (Ag) coating with HA composites which contain ATM biomaterials in bone cements or gentamicin-loaded Polymethylmethacrylate (PMMA) bone cements (Dian et al., 2009; Neut et al., 2010).

1.3 Mangrove Plants

Natural sources such as plants are good sources for ATM chemotherapeutic agents (Saad et al., 2011). It has been recognized that the naturally occurred substance in higher plants have the potential to be used as the ATM agents. Furthermore, scientist and research reported the higher plants produce hundreds to thousands diverse of chemical compound with different biological properties and also important in ecological roles. ATM compound that produced by the plant are very active against the human pathogen (Lim et al., 2006). In a view for smaller scope of ATM agents that extracted from natural sources, mangrove plants was selected for this research study as Malaysia was largely surrounded by the mangrove forest (Lim et al., 2006; Saad et al., 2011).

Malaysia mangroves forests are unique ecosystem that generally found along sheltered coasts where they grow abundantly in saline soil and brackish water subject to periodic fresh and salt-water inundation. Mangrove trees have specific characteristics such as tough root systems, special bark and leaf structures and other unique adaptations that enable them to survive in their habitat's which in harsh conditions. Mangrove habitat are soft, silty and shallow, coupled with the endless ebb and flow of water providing very little support for most mangrove plants which have aerial or prop roots which known as pneumatrophores, or respiratory roots and buttressed trunks (WWF-Malaysia, 1972).

1.4 Problem Statement

Deep infections in a total joint replacement are potentially catastrophic events for patients (Torrado et al., 2001). Infections in total joint arthroplasty are devastating situations, and many strategies have been taken to reduce the infection rates, including the use of helmet aspirator suits, laminar airflow, and prophylactic intravenous antibiotics (Alt et al., 2004, Torrado et al., 2001). Furthermore, Sandiford et al. was reported more than 80% of infections are recognized and certainly the majority of those infection by involving from the medical devices during surgery (Sandiford et al., 2010).

Therefore, by having antibiotic-loaded bone cement in bone replacement procedure, it will fix the implants within antibiotic acting to reduce the risk of infection. Otherwise, Sandiford et al. was suggested to have a biofilm element of antibiotic for the surgical development. In different meaning, it involves the incorporation of antimicrobial peptides directly into medical devices and materials which can prevent the development of potentially bacteria as a dangerous biofilm (Sandiford et al., 2010).

Sometimes in a clinical surgery, surgeons have been mixed the antimicrobial into bone cement, but the mixing of antimicrobial is intra-operatively to bone cements formulas and will present certain risks. For examples, the surgeon can never be sure that antibiotic is evenly distributed throughout mixture or mechanical properties of bone cement cannot be compromised in terms of ATM efficiency (Torrado et al., 2001).

Moreover, new ideas in orthopedics implantation should be evaluated stepwise with in vitro and subsequently in vivo investigation of ATM properties occurring before the clinical trials. Therefore, testing the ATM activities of bone cement with new antibiotics or other ATM agents should begin with in vitro study (Alt et al., 2004). Antibiotic-loaded cement also should be considered as a defense against directly

contamination during the surgery or the postoperative period as the wound seals (Suman et al., 1986). However, the role on preventing the infection remains controversial because of some issues regarding drug resistance, efficiency and cost.

Currently, synthetic source of antimicrobial drug may gives a negative side effect. For examples, increase of the opportunistic infection and the effect for the human health by continuous uses of several antimicrobial drugs, are several of side effects to human body. These affect also may harm the human body in long term duration (Saad et al., 2011). However, there have another ways to overcome these side effects which is by having necessitated research for a new ATM agent from natural sources like a plants. This is because plants are good sources of novel ATM chemotherapeutic agents (Saad et al., 2011). Plants have been a major source in drug development. Even today, plant materials are continued to play a major role in primary health care as therapeutic remedies in many developing countries. In addition, the products from plant extraction are widely used in the treatment of infectious disease (Saad et al., 2011).

According to the current views on antimicrobial drugs, natural substances with fewer synthetic additives hold a better confidential element due to the safety and shelf-life in human health (Negi et al., 2008). Resulting from those demands, plants emerged as an important and popular ingredient which has a tendency of replacing synthetic ATM agents (Negi et al., 2008).

In addition, Mangrove plants are one of the natural products that can be selected for biological screening regarding on the herbal medicines. This is because of many infectious diseases are known to be treated with herbal remedies throughout the historical of mankind. In addition, mangrove plants contain biologically active antiviral, antibacterial and antifungal compounds (Chandrasekaran et al., 2009). These mangrove