SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES DERIVED FROM *Pleurotus sajor caju* AND ITS BIOLOGICAL ACTIVITY ON *Candida albicans*

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BY

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I dedicate this thesis to my beloved father, Musa B. Malek and my mum, Napsiah Binti Ismail for their unconditional love and support throughout my life. To my brothers and their family, Mohd Fadhil, Mohd Firdaus, Mohd Fariad and Mohd Fikri who always be there for me. Thank you for making me who I am today.

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LIST OF SYMBOLS AND ABBREVIATIONS

AgNPs	Silver nanoparticles
ICL1	Isocitrate lyase gene and enzyme
MLS1	Malate synthase gene and enzyme
RT-qPCR	Quantitative real time polymerase chain reaction
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
Ag	Silver
Zn	Zinc
Fe	Iron
Au	Gold
Nm	Nanometer
$AgNO_3$	Silver nitrate
A431	Human skin carcinoma cells
HT-1080	Human fibrosarcoma cells
HaCaT	Human skin keratinocyte
mg/L	Milligram per liter
FTIR	Fourier Transform Infrared
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
%	Percentage
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide

cAMP-KA	Cyclic AMP- mitogen-activated protein (MAP) kinase pathway
ECM	Extracellular matrix
PEP	Phosphoenolpyruvate
NADH	Nicotinamide Adenine Dinucleotide – Hydrogen
TCA	Tricarboxylic acid
$FADH_2$	Flavin adenine dinucleotide-hydrogen
GFP	Green fluorescent protein
PCK1	Phosphoenolpyruvate carboxykinase 1
VCO	Virgin coconut oil
mRNA	Messenger ribonucleic acid
TBE	Tris/Borate/EDTA
EDTA	Ethylenediaminetetraacetic acid
mL	Mililitre
^oC	Degree Celsius
g	Gram
x g	Relative centrifugal force
µm	Micrometre
mM	Milimetre
TEM	Transmission electron microscopy
KBr	Potassium Bromide
XRD	X-ray diffractometer
YPD	Yeast-peptone-dextrose
YPL	Yeast-peptone-lactate

cfu/mL	Coloning form unit per millilitre
Ω	Ohm
RNA	Ribonucleic acid
Kb	kilo- base pair
µL	Microliter
cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
gDNA	Genomic deoxyribonucleic acid
NaCl	Sodium chloride
RIPA	Radioimmunoprecipitation assay
BSA	Bovine serum albumin
µg/mL	Microgram per mililitre
Na_2HPO_4	Disodium phosphate
NaH_2PO_4,	Monosodium phosphate
mM	Mili molar
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
ECL	Enhanced chemiluminescence
mV	Mili volt
Θ	Theta
kDa	kilo Dalton
SPR	Surface plasmon resonance

JCPDS	Joint Committee on Powder Diffraction Standards
ICDD	International Centre for Diffraction Data®

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SINTESIS DAN PENCIRIAN NANOPARTIKEL PERAK OLEH *Pleurotus sajor caju* DAN AKTIVITI BIOLOGINYA KE ATAS *Candida albicans*

ABSTRAK

Nanopartikel perak (NP) telah dikaji secara meluas kerana keupayaannya sebagai anti-kulat dan anti-bakteria. Kaedah hijau untuk menghasilkan NP telah digunakan secara intensif kerana proses penghasilannya yang mesra persekitaran serta tidak menggunakan agen penurun yang toksik. *C. albicans* merupakan salah satu kulat yang boleh direncatkan pertumbuhannya oleh NP. *C. albicans* menjadi virulen kerana ia mempunyai keupayaan metabolik untuk hidup dalam kondisi pertumbuhan yang berbagai. *C. albicans* mempunyai kebolehan untuk hidup dalam kondisi ketiadaan atau kepekatan glukosa yang rendah. Bagi memastikan kelangsungan hidup, ia perlu meggunakan sumber karbon yang tidak fermentasi seperti asid laktik dan diproses melalui kitaran glioksilat manakala isositrat liase (Ca*ICL1*) merupakan gen yang terlibat dalam kitaran tersebut. Namun demikian, gen ini merupakan salah satu penyumbang kepada bahayanya *C. albicans*. Oleh itu, dalam kajian ini, NP yang terhasil dengan menggunakan ekstrak *Pleurotus sajor caju* atau dikenali sebagai cendawan tiram kelabu telah dikenalpasti setelah penukaran warna daripada kuning pucat ke oren kemerahan selepas 72 jam inkubasi. Purata saiz diameter partikel adalah 11.68 nm serta potensi zeta -8.54 mV. Spektra daripada FTIR telah menunjukkan bahawa protein serta polisakarida yang terdapat dalam ekstrak cendawan bertanggungjawab untuk menurunkan ion perak kepada NP. Spektra daripada XRD juga menunjukkan bahawa partikel yang terhasil adalah dalam bentuk ‘face centred cubic’. Bagi mengenalpasti aktiviti anti-kulat oleh NP, kaedah cairan mikro-pencairan telah digunakan. Hasilnya, NP menunjukkan nilai MIC adalah 250

mg/L dan MFC adalah 500 mg/L. Impak NP terhadap CaICL1 di peringkat gen serta protein telah dikenalpasti melalui kaedah kuantitatif PCR (qRT-PCR) serta analisis western blot. Oleh itu, keputusan eksperimen ini menunjukkan ekspresi Ca*ICL1* yang diinkubasi bersama NP berkurang pada jam ke-2 dan ke-4 manakala pada jam ke 6 eksperesinya bertambah melepasi kontrol. Ekspresi CaIcl1 di dalam sel yang telah diuji dengan NP dan tanpa NP menunjukkan pengurangan setelah 2 jam inkubasi tetapi bertambah pada jam ke-4 dan ke-6 inkubasi. Hal ini kerana, NP dicadangkan dapat membantutkan pertumbuhan *C. albicans* tetapi tidak mengurangkan ekspresi isositrat liase dan asid laktik masih digunakan. Pembantutan itu kemungkinan disebabkan oleh kebolehan sel untuk mengadaptasi tekanan osmosis terjadi akibat NP melalui pertahanan dinding selnya.

SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES DERIVED FROM Pleurotus sajor caju AND ITS BIOLOGICAL ACTIVITY ON Candida albicans

ABSTRACT

Silver nanoparticle (AgNPs) is widely studied due to its antifungal and antimicrobial activities against a wide range of microorganisms. In previous study, *C. albicans* is one of the pathogenic yeast that has been reported to be inhibited by AgNPs. Green method used to synthesise AgNPs also has been intensively studied due to its environmentally friendly and the used of non-toxic reducing agent. Meanwhile *C. albicans* was found to be virulent due to its metabolic flexibility to survive in a wide range of growth conditions. It has the ability to grow in the absence or in low glucose concentration. In order for yeast to survive, it must assimilate the non-fermentable carbon sources such as lactate instead of glucose through glyoxylate cycle and the isocitrate lyase (Ca*ICL1*) is one of the genes involved in this cycle. Plus, the expression of the gene was reported to be one of the factors contributed to *C. albicans* virulence. In this study, AgNPs synthesised using *Pleurotus sajor caju* mushroom or its common name grey oyster mushroom from hot water extraction was used and it has been confirmed to form AgNPs by the color changed from pale yellow to reddish brown after 72 hours of incubation with 1mM $AgNO_3$. The mean particle size of AgNPs obtained was 11.68 nm with the zeta potential value -8.54 mV. The FTIR spectra also showed that the protein and polysaccharide in. *P. sajor caju* extract was responsible in reducing silver ion to silver nanoparticles. Meanwhile, XRD spectra also showed that the nanoparticle is the face-centred cubic (fcc) structure of silver.

Therefore, the broth microdilution assay was carried out to determine the antifungal activity of AgNPs. As a result, the minimum inhibitory concentration (MIC) of AgNPs was 250 mg/L while the minimum fungicidal concentration (MFC) was 500 mg/L. The impact of AgNPs on CaICL1 at the transcription and protein level also was observed in this study through quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. The result showed that the expression of Ca*ICL1* was downregulated at 2 and 6 hours incubation and then it was upregulated after 6 hours incubation. Meanwhile, CaIcl1 expression in the treated cells decreased after 2 hours and then increased after 4 and 6 hours compared with untreated time which was reduced after 6 hours incubation. Thus, this result suggested that AgNPs was able to inhibit *C. albicans* growth, but it was not able to repress isocitrate lyase production which is needed for lactate utilization. Hence, the inhibition of *C. albicans* by AgNPs also occurred at certain period of time might be due to the ability of *C. albicans* to adapt to osmotic and heavy metal stress caused by the AgNPs.

CHAPTER ONE: INTRODUCTION

Silver nanoparticles (AgNPs) are defined as particles with the diameter size less than 100 nm and it can be synthesised through chemical, physical or biological method (Guzman *et al.* 2012; Krishnaraj *et al*., 2010; Jung *et al*.,2006). Biologically synthesised AgNPs has received much interest among researchers recently, due to its environmental friendly, economical and does not use or produce any toxic chemicals during synthesis process (Mie *et al.*, 2013). Various natural sources involved in this method such as plant, bacteria and fungus. A higher fungus, such as mushroom has been widely used in this method due to the bioactive compound present in the extract such as vitamin, protein and polysaccharide. These compounds serve as reducing agents and natural stabilizers to produce AgNPs from Ag^{+} (Carbonero *et al.,* 2012; Pramanik *et al*., 2005).

In addition, various species of mushroom extract have been used as natural reducing agent such as *Pleurotus spp, Ganoderma lucidum, Agaricus bisporus* and *Tricholoma crissum* (Raman *et al*., 2015; Karwa *et al*., 2011; Narasimha *et al.*, 2011; Ray *et al.*, 2011). *Pleurotus sajor caju* was reported to have higher polysaccharide content and protein in its extract (Pramanik *et al*., 2007; Pramanik *et al*., 2005). The bioactive compound present has antifungal and anti-tumor properties towards fungus and tumor cells respectively (Finimundy *et al*., 2013; Kanagasabapathy *et al*., 2011; Ngai and Ng, 2004). *P.sajor caju* is widely cultivated in Malaysia and the short production period makes it available throughout the year. Therefore, it was considered to be used in synthesis of AgNPs due to the reasons stated earlier.

Furthermore, the AgNPs is widely used in pharmaceutical and biomedical applications. This is due to its small particle size that can enhance its antifungal activity by providing high surface area to volume ratio of the particles to the cells. Meanwhile previous studies reported that the AgNPs has positive inhibition towards *C. albicans* which is opportunistic yeast that normally present in healthy human with commensalism interaction with the host. However, in stress condition, it can be virulent and cause candidiasis infection to the host (Lorenz *et al*., 2004). Normally, people with weak immune system have a high possibility to be infected for example after chemotherapy procedures, post-organ transplant and patients infected with human immunodeficiency virus (HIV) (Neofytos *et al.*, 2010; Neofytos *et al*., 2013; Egusa *et al*., 2008). Besides, it also can cause a mild infection to pregnant woman and diabetes mellitus patient (Guzel *et al*., 2011; Tsang *et al*., 2007).

Additionally, people who have been exposed regularly to antifungal drugs also can have candidiasis infection. It is because mutated *C. albicans* have become resistant towards the drugs such as azole group and it was no longer effective to inhibit the yeast growth at susceptible concentration (Spampinato and Leonardi, 2013). Besides, *C. albicans* grown in non-fermentable carbon source also contributed to its virulence due to the gene regulation that enable yeast to switch its phenotype from budding yeast to hyphae for cell penetration either forming biofilm on cells or escape from the macrophage.

Therefore, to my knowledge the mechanism of inhibition of AgNPs towards *C. albicans* is still unclear. Thus, this study was carried out to determine the impact of inhibition at the molecular level of *C. albicans* treated with AgNPs which was

synthesised using *P. sajor caju* extract. Hence, the isocitrate lyase enzyme, Ca*ICL1* was selected due to its function in the activation of glyoxylate cycle. The glyoxylate cycle was required by *C. albicans* to utilize the non-fermentable carbon sources such as lactate to generate glucose for energy and cell function. It was normally available in several niches such as skin, gastrointestinal tract and mucosal membrane where glucose was limited or not available (Calderone and Fonzi 2001). Unfortunately, glyoxylate cycle was reported to be one of the factors that contribute to *C. albicans* virulence. As a result, this study was conducted in order to provide a brief understanding of the mechanism of AgNPs inhibition towards the *C. albicans* at transcription and protein level and the CaICL1 served as the potential antifungal drug target.

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Objectives of the project

1. To synthesise and characterise silver nanoparticles from the extract of *Pleurotus sajor caju* .
2. To determine the minimum inhibitory concentration (MIC) and minimum fungicidal inhibition (MFC) of silver nanoparticles towards *C. albicans* in lactate medium.
3. To study the impact of silver nanoparticles on *C. albicans* isocitrate lyase Ca*ICL1* transcription level.
4. To evaluate the impact of silver nanoparticles on isocitrate lyase CaIcl1 at protein level .

CHAPTER TWO: LITERATURE REVIEW

2.1 Metal nanoparticles

Synthesis of nanomaterial such as metal nanoparticles for biological purposes is part of the nano-biotechnology application. Generally, nanoparticles can be synthesised through two different approaches, either reduction of size from suitable bulk material (top down) or by the accumulation of the atoms and molecules strengthened by chemical bond to form nanoparticles (bottom up). The top down synthesis involves the physical method such as the use of tube furnace with laser and ceramic heater (Jung *et al*., 2006). Meanwhile bottom up synthesis involves the use of chemical solvent such as borohydrate, citrate and ascorbate (Guzman *et al*., 2012), as well as by using natural biological sources intra and extracellular compounds to form nanoparticles.

However, both chemical and physical methods have several limitations such as high energy consumption, less economical and high possibility to release toxic by-products to the environment (Thakkar *et al.*, 2010; Das and Thiagarajan, 2012). Therefore, biological or green synthesis method was proposed to be used in order to overcome the existing limitations because the process involved is environmental friendly, rapid, economical and suitable for large-scale production. Moreover, this method of synthesis can be monitored by optimizing the pH, concentration of reducing agent, and temperature. The natural reducing agents commonly used to synthesis metal nanoparticles derived from plant (Krishnaraj *et al.*, 2010), bacteria (Shahverdy *et al*., 2007), and fungi (Ahmad *et al*., 2003).

A lot of studies have been performed to determine the potential of using metal nanoparticles in various applications due to their small size which will provide high surface area to volume ratio, good electrical conductivity and toxic to wide range of microorganism. Hence, the metal such as silver (Ag) (Saxena *et al.,* 2012), zinc (Zn) (You *et al.*, 2011), copper (Cu) (Cronholm *et al.*, 2013) iron (Fe) (Mazumdar and Haloi, 2011) and gold (Au) (Cui *et al.*, 2012) are the common metal used to synthesise nanoparticles.

Among various metals, noble silver (Ag) has received much attention to be used in synthesis of metal nanoparticles. For example, in household products, silver nanoparticles (AgNPs) was used embedded in ceramic coating of water filters (Das *et al.,* 2012) and activated carbon air filters (Yoon *et al.,* 2008) to prevent bacterial and fungal colonisation. It was used as component in food packaging, tableware and kitchenware (Chaudhry *et al*., 2008). Apart from that, AgNPs was also used in formulated shampoo as an anti-dandruff (Pant *et al*., 2013), as a preservative in cosmetic products (Kokura *et al*., 2010) and also in formulated ultrasound and wound healing gel (He *et al*., 2013; Jain *et al*., 2009). Besides, AgNPs also was used in antibacterial finish on fabrics such as socks, laboratory gown and sports clothing (Vankar and Shukla, 2011).

On top of that, it was also used as one of the important component in various medical tools such as coating pin for surgical practice, bandage, cavity filler, in bone cement, catheter and wound dressing (Chaloupka *et al.,* 2010; Arora *et al.,* 2008; Alt *et al.*, 2004). It is very useful to prevent bacterial colonisation and dissemination without harming the cells spreading for recovering process

(Maneerung *et al*., 2008; Bosetti *et al*., 2002). Other study showed that AgNPs also can be considered to be applied as an alternative mosquito's vector control due to the positive inhibition against larvae of *Anopheles stephensi*, *A. subpictus*, *Cule quinquefasciatus* and *Aedes aegypti* which can cause malaria and dengue fever (Suganya *et al.*, 2013; Marimuthu *et al*., 2011).

2.1.1 Silver nanoparticles: Is it safe?

Widely exposed to AgNPs has created major concern among researchers due to its toxicity effect on human and environment. Recently, the effect of AgNPs on environment was highly debated due to the AgNPs detected in aquatic life and animal lives nearby. It was reported that, aquatic organism such as fathead minnow fish and oyster embryos (Laban *et al.*, 2010; Ringwood *et al*., 2010), adult zebrafish (Choi *et al.*, 2010) and rainbow trout (Scown *et al.*, 2010) showed positive AgNPs uptake upon exposed to different concentration of AgNPs. The release of Ag^{+} and AgNPs to the environment most probably from the wastewater produced from textile and plastic industry. However, the AgNPs uptake detected in previous studies was due to the direct expose of AgNPs in a tank that mimic the real condition of their habitat. Therefore, this may exhibit bias result compared with real sampling due to changes in AgNPs concentration and effective treatment of the wastewater before it released to freshwater system (Blaser *et al.*, 2008).

On the other hand, *in vitro* and *in vivo* studies also were carried out to detect any adverse effect to human health upon exposure to the AgNPs. Previous study showed that, AgNPs exhibit high toxicity effect to the human skin carcinoma cells

(A431). Hence, the positive effect of AgNPs to anti tumor leads to the investigation on the impact of AgNPs towards the normal cells. Therefore, AgNPs tested on human fibrosarcoma cells (HT-1080) (Arora *et al*., 2008) and human skin keratinocyte (HaCaT) cells (Mukherjee *et al.,* 2012) showed that it was toxic to the cells at low concentration, less than 50 mg/L. The cells were dead mainly due to the generation of reactive oxygen species (ROS) and cells apoptosis. Meanwhile, the toxicity of AgNPs *in vitro* is highly depended on the concentration of AgNPs, particle size and the tendency of AgNPs to release free silver ion (Ag^{+}) (Xiu *et al*., 2012).

In contrast, the AgNPs showed less toxic in clinical testing (*in-vivo).* Previous study showed that AgNPs was found in healthy human skin after the topical cream impregnated with AgNPs was applied on them (Larese *et al*., 2009). However, the level of penetration considered safe because the AgNPs was found only at the hypodermis layer and it was not penetrate the systemic circulation which also means no organ will be affected (Stees *et al*., 2015; George *et al*., 2014). Furthermore, AgNPs also has high potential to be used as preservative in cosmetic product and promote wound healing until it closely resemble with the normal skin (Kwan *et al*., 2011; Kokura *et al*., 2010; Tian *et al*., 2007). In other study, the release of Ag^{+} from fabric and textile impregnated with AgNPs into artificial sweat showed that it strongly depend on the initial concentration of AgNPs used and the pH of the sweat (Kulthong *et al*., 2010). Thus the AgNPs synthesis process was assumed not to cause adverse effect to the environment unless the AgNPs waste water was poorly monitored. Plus, the AgNPs toxicity to human health also is solely depend on the mode of application of AgNPs .

2.1.2 Silver nanoparticle synthesised from fungi

Previously, various fungi species including ascomycete and basidiomycete were used to synthesise AgNPs. For example, the intracellular and extracellular products of *Trichoderma viride* (Fayaz *et al*., 2009), *Aspergillus terreus* (Rekha and Arya, 2013). Meanwhile, in mushroom, the aqueous extract of the fruiting body, mycelium, mycelium broth and mushroom's spent or substrate (Bhat *et al*., 2011; Ray *et al*., 2011) was majorly used in AgNPs synthesis process (Gurunathan *et al.*, 2013; Karwa *et al*., 2011). In addition, the mushroom extract has rich nutritional biomolecule compound such as polysaccharide including α and β-glucan (Shenbhagaraman *et al.,* 2012), peptide such as pleurostrin, lentin and hypsin (Chu *et al.*, 2005; Ngai and Ng, 2003; Lam and Ng, 2001), flavonoid as well as ascorbic acid (Vamanu, 2012) that can act as natural stabilizer and reducing agent to form AgNPs.

Therefore, various mushroom species have been used in synthesis of AgNPs as shown in Table 2.1. Instead of high nutritional content, synthesis of AgNPs from the extract also was chosen due to the involved of environmental friendly process, short harvesting period, non-pathogenic and no toxic by-product produced that make it safe and economical to be served as reducing agent. Plus, the mushroom's cultivation can be monitored to produce healthy mushroom, short harvesting period, free pesticide and also less space needed. In fact, the extract can be obtained from both stages of mushroom; mycelium mushrooms (Sugihara and Humfeld, 1954; Kim *et al*., 2003; Elisashvili 2012; Papaspyridi *et al*., 2012) and its fruting body (Vigneshwaran *et al*., 2007).

Table 2.1 : Various species of mushrooms used to synthesized silver nanoparticles.

Mushroom species	**References**
Pleurotus djamor var. *roseus*	(Raman et al. 2015)
P. cornucopiae var. *citrinopileatus*	(Owaid et al. 2015)
Lentinus squarrosulus (Mont.) Singer	(Manna et al. 2015)
Metharhizium anisopliae	(Amerasan et al. 2015)
G.neo-japonicum Imazeki	(Gurunathan et al. 2013)
Schizophyllum radiatum	(Metuku et al. 2013)
P. djamor	(Shivashankar et al. 2013)
Ganoderma lucidum *(W.Curt.:Fr.) P.Karst*	(Karwa et al. 2011)
Agaricus bisporus	(Narasimha et al. 2011)
Tricholoma crassum	(Ray et al. 2011)
Pleurotus florida	(Ipsita Kumar Sen et al. 2013) (Bhat et al. 2011)
P. sajor caju	(Nithya and Ragunathan, 2009)
Volvariella volvacea	(Philip, 2009)

The *P. sajor caju* or commonly known as oyster or abalone mushroom is an edible mushrooms contained high nutritional value such as vitamins, protein, fibres, minerals and polysaccharide. In fact, it has been consumed and used as medicine for many years before it was extensively studied for several treatments and applications. Previous study showed that carbohydrate is the major compound present in the *P. sajor caju* which is ~37- 40 g/ 100 g of mushroom and then followed by protein and fiber content (Khan and Tania, 2012). Generally, mushroom consist of three layers made up of glucan. The outer layer contained water soluble glucan, while second and third layer contained alkaline soluble glucan (α-(1→3)-glucan) and alkaline insoluble glucan (β-(1→3)-glucan) respectively (Satitmanwiwat *et al*., 2012). Recent studies found that, D-glucose, D-galactose, D-mannose and D-fructose are the main component present in extract of *P. sajor caju* (Pramanik *et al.,* 2007; Pramanik *et al*., 2005; Roy *et al.*, 2008).

The glucan was effective as an antitumor and anti-inflammatory agent *in vivo* (Silveira *et al.*, 2014; Dalonso *et al.*, 2010). Hence, it has the potential to be used in diabetes mellitus treatment due to its ability to activate AMPK (AMP-activated protein kinase) which is the enzyme responsible for lipid and glucose metabolism for energy production (Kanagasabapathy *et al*., 2014). Glucan was used to synthesis AgNPs due to its function as excellent template for nucleation and stabilization of nanoparticles (Sen and Islam, 2013). In fact, the extract also has abundance of hydrophilic hydroxyl groups and soluble in water which make it suitable to be used in synthesis of AgNPs (Sen *et al.*, 2013).

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2.1.3 Silver nanoparticles: Mechanism of action

The AgNPs was reported to have antimicrobial activity against wide range of bacteria and fungus. Thus, the growing interest in implementing AgNPs in various applications has led to the necessity to understand the mechanism of AgNPs inhibition during treatment. In previous studies, the observation under electron microscope showed that the cells were dead due to the accumulation of AgNPs at the microbial cell wall (Vazquez-Muñoz *et al*., 2014; Ahmad *et al.*, 2013; Monteiro *et al.*, 2013; Mirzajani *et al*., 2011; Li *et al.*, 2010). In fact, formation of pits and pores at the cell membrane has increased its permeability thereby the small particles were detected in the cells cytoplasm (Sondi and Salopek-Sondi, 2004).

However, AgNPs was claimed to be no longer effective to inhibit microbial due to inability to release free (Ag^{+}) during treatment. This is because the key mechanism of AgNPs toxicity is the availability of particles to release Ag^{+} during treatment. This theory was proven by comparing the effect of AgNPs on bacteria in aerobic and anaerobic incubation with different particles size. As a result, AgNPs in anaerobic chamber showed negligible toxicity effect towards bacteria due to the absence of oxidizing agent to release Ag^{+} and the particle size did not have any major impact on its toxicity level (Xiu *et al*., 2012). Therefore, the ability of AgNPs to release free silver ion (Ag^{+}) due to the natural oxidation process during antimicrobial test was proposed to be the one of factor contributed to the AgNPs toxicity and the oxidation process occurred mainly due to the dissolved oxygen and protons present in the growth medium (Sotiriou *et al.*, 2012; Xiu *et al.*, 2012).

Reduction in AgNPs size possesed high antimicrobial activity due to the high surface area to the volume ratio and high tendency for smaller AgNPs to release free Ag^+ faster compared with the large size of AgNPs (Vazquez-Muñoz *et al*., 2014; Xiu *et al.,* 2012). Other than that, the stabilizer used in synthesis of AgNPs also led to the high toxicity of AgNPs. The stabilizers such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG) and polyvinyl alcohol (PVA) were used to prevent agglomeration within AgNPs thus reduce the tendency to form large particles (Nabikhan *et al.,* 2010; Selvaraj *et al.,* 2014). The stabilizer also gives an effect to surface composition to the AgNPs. For example, AgNPs with the oxide layer on the surface has high affinity to interact with the cells and thus causing cells damaged (Sotiriou *et al*., 2012).

Consequently, recent study reported that the cellular response of mammalian cell has significant impact to cell death upon expose to AgNPs. This is supported by other study where the human cancer cell line was dead due to the Ag^+ ion released in cytosol which means the the oxidizing process occurs inside the cells (De Matteis *et al*., 2015). Hence, the ion released in the cells also was due to the oxidizing process by hydrogen peroxide present in the macrophage (Loza *et al.,* 2014). The free Ag^+ reacted with other sulphur-containing proteins in the cytoplasm as well as DNA which consist of phosphorus compound followed by protein inactivation and DNA malfunction (Rahisuddin e*t al.,* 2015; AshaRani *et al*., 2009).

2.2 Candidiasis

Candidiasis is a disease caused by fungal infection and it is mainly affected by *Candida* species such as *C. albicans, C. glabrata, C. lusitaniae, C. framata, C. krusei, C. parapsilosis* and *C. dubliniensis*. It can cause non-life threatening to invasive infection and most of the time the infections occured at distinct niches such as mucosal membrane, skin, gastrointestinal tract and vagina (Brown *et al*., 2014). As a matter of fact, the presence of *C. albicans* is normal in 30-70% of healthy people but it can be pathogenic to those who has weak immune system due to the inability to control the *C. albicans* colonisation (Gow *et al*., 2012). Therefore, the candidiasis infection isolated from patients in Malaysia has been monitored every year in order prevent outbreak in fungal infection. In previous year, the Department of Medical Microbiology in University of Malaya reported that *C. albicans* was the third major *Candida* species isolated from blood sample and it remained constant in number throughout the year (Ng *et al.,* 2001).

Hence, in other report, *C. albicans* become a major *Candida* species isolated from various clinical samples such as from vagina, blood, peritoneal, dialysis fluid, urine and cerebrospinal fluid (Ding *et al*., 2014; Chong *et al*., 2003). In 2011, several strains were found to be resistant towards commercial antifungal drug, fluconazole (Amran *et al*., 2011). Plus, in 2013, *C. albicans* (clinical isolate) showed greater virulence potential due to its high phospholipase and haemolysin activity (Chin *et al*., 2013) compared to non- *C. albicans*.

2.2.1 Current studies on candidiasis

In present time, several commercial antifungal drugs were used to control candidiasis such as azoles, polyene and echinochandin. Most of these antifungal drugs inhibit *C. albicans* via perturbation of ergosterol synthesis pathway. Meanwhile, the posaconazole, fluconazole and voriconazole were the subset of azoles groups and despite fluconazole, all of them were successfully used since 1960s (Odds *et al*., 2003). The mechanism of inhibition by azoles is through the inhibition of 14α-demethylation of lanosterol which is one of the main components of cytochrome P450 in fungal membrane. The inhibition was due to the ergosterol depletion and accumulation of toxic compound, methylated sterol intermediated (Delattin *et al.*, 2014). Azole has been used widely due to the antifungal activity against broad range of fungus and less expensive compared with other commercial drugs (Spellberg *et al.*, 2006).

Besides, amphotericin B and nystatin are the subset in polyene group. It will cause cell death by binding to the ergosterol present in fungal cell membrane. It increases membrane permeability through the formation of pore thus causing cellular component leakage. However, this group has some limitation which is low selectivity because it can also bind to the ergosterol present in other mammalian cells. Lastly, caspofungin, micafungin and anidulafungin were from the echinochandin group. It inhibit fungal growth by inhibit the protein responsible for β-1,3 glucan synthesis which is important in cell wall development. β-1,3 glucan also was found to be present in matured extracellular matrix (ECM) or biofilm of *C. albicans*. Therefore, echinochandin drug also can prevent the drug resistance caused by *C. albicans* biofilm (Bachmann *et al*., 2002).

However, the consumption of commercial antifungal drugs including azole, polyenes, echinocandins and allylamine groups for such a long time lead to antifungal drug resistance in *C. albicans* (Spampinato and Leonardi, 2013; Priya and Pei Pei, 2011). Therefore, there is a need to develop alternative antifungal agent from biological compound to combat with *C. albicans* infection. In 2009, studied on plant based compounds; cinnamaldehyde, piperidin, indole, furfuraldehyde and citral showed high antifungal activity against *C. albicans* where citral and furfuraldehyde have the highest percentage of ergosterol reduction which is 96% and 96% respectively (Rajput and Karuppayil, 2013).

In fact, a lot of studies were done to find alternative approaches to treat candidiasis which are mainly focusing on biological sources such as virgin coconut oil (VCO) and other spices like cinnamon and pogostemon. As a result, they have been identified to have antifungal activity against *C. albicans* with the comparable susceptibility with antifungal drug tested, fluconazole (Ogbolu *et al*., 2007; Wang *et al*., 2012). In other studies, novel mixture of bee-honey, yogurt, aloe vera and edible mushroom also was found to be one of the useful treatment for vulvovaginal candidiasis (VVC) patients (Abdelmonem *et al*., 2012; Bernardes *et al*., 2012; Vamanu, 2012). Thus, natural herb has great potential in reducing the used of synthetic drug for fungal infection treatment.

2.2.2 Factors affect the pathogenicity of *C. albicans*

C. albicans is an opportunistic yeast and it has the ability to colonise human tissues and organ. Therefore, to survive in various niches, *C. albicans* must be able to adapt to different conditions that might cause cell stress. However, its capability to regulate the heat shock, osmotic and oxidative stress promoted *C. albicans* pathogenicity. Plus, remodelling of cell wall structure, the ability to change its morphology and enhance the formation of biofilm through adherence and secretion of hydrolase enzyme also can cause virulence in *C. albicans.*

The heat shock response is needed by *C. albicans* to adapt with drastic changes in temperature. Different in temperature can trigger the protein unfolding and aggregation of non-specific protein in *C. albicans* (Mayer and Hube, 2013; Brown *et al*., 2014). Therefore in order to survive, the heat shock factor (Hsf1) will regulate the heat shock proteins (HSPs) to refold and degrade the damaged proteins for cell new temperature adaptation (Leach *et al*., 2012; Nicholls *et al*., 2009). However, HSPs also can contribute to *C. albicans* pathogenicity in biofilm formation, yeast-hyphae development and activation of Cek1 signalling (Fu *et al.*, 2012; Mayer *et al.*, 2012; Brown *et al*., 2010). In fact, *C. albicans* with malfunction of Hsf1 shows attenuate virulence in a mouse model due to inactivation of HSPs (Nicholls *et al*., 2011).

Additionally, the *C. albicans* was found to be able to survive in innate immune system; macrophage and neutrophils. To achieve that, it must be able to adapt with oxidative stress which was due to the accumulation of reactive oxygen species (ROS) such as, superoxide anion, hydrogen peroxide and hydroxyl radical (Jamieson *et al*., 1996) in the immune cells. In healthy people, the ROS in macrophage is responsible to destroy microbe through respiratory burst process (Dantas *et al*., 2015). However, in immunodeficiency people, *C. albicans* is able to survive in oxidative stress condition using three signalling pathways that were shown to activate the response to ROS. They are Cap1 transcription factor, Hog1 stress-activated protein kinase and lastly Rad53 DNA damage check point kinase (Dantas *et al*., 2010; Gónzalez-Párraga *et al*., 2010).

Other than that, *C. albicans* cell wall is important to maintain its osmotic balance. Perturbation in osmotic balance triggered the activation of stress-activated protein kinase (SAPKs). Then, the protein stimulated the genes encode for glycerol biosynthetic pathway like *HOG1* which is important for cells survival and glycerol accumulation (Enjalbert *et al*., 2006; Smith *et al*., 2004). However, recent study showed that, Hog1 and glycerol accumulation were required for osmoadaptation but not for *C. albicans* survival (Ene *et al.*, 2015).

Besides, osmotic stress also can be due to the utilization of non-glucose carbon source such as lactate. *C. albicans* grew in lactate showed alteration at the cell wall structure thus promote osmotic stress to the cells (Ene *et al.*, 2012). On the other hand, *C. albicans* grew in lactate was more resistant to hyperosmotic shock than cells grown in glucose because it promote the increased of cell wall elasticity

(Ene *et al.*, 2015). In other report, glycerol 3-phosphatase (Gpp1), glycerol 3-phosphate dehydrogenase (Gpd2) and Sko1 transcriptional factor also involve in glycerol biosynthesis mechanism which is required to synthesis glycerol after loss of water due to the difference in osmotic pressure (Wächtler *et al*., 2011; Rauceo *et al*., 2008). Thus, it can be concluded that *C. albicans* remodelling of cell wall structure is strongly related with the adaptation of osmotic stress for cell survival.

C. albicans cell wall consists of two main layers; inner and outer cell wall and comprised with several components as shown in Figure 2.1. Those are mannan, proteins, β-1,6-glucan, β-1,3-glucan and chitin which are strengthening by covalent bond, hydrogen bond, salt-type associations, hydrophilic and hydrophobic interactions. All components serve as protector against physical, chemical and biological harm towards the cell. However, some components in *C. albicans* cell wall plays important role in its pathogenicity (Ruiz-Herrera *et al*., 2006). For example, *C. albicans* cell wall hydrophobic area is important for its biofilm formation and it depends on polysaccharide at the outer layer of cell wall which is acid labile β-1,2-oligomannosides (Hobson *et al*., 2004).

Its hydrophobic property is important in biofilm formation and it has been identified by using microarray assay and after treated with farnesol (compound used to inhibit biofilm formation). As a result, the cell surface hydrophobicity-associated gene (CSH1) were found to be associated with the hydrophobic properties of the cells (Cao *et al*., 2005). Besides, other glycoproteins like *CSF4*, GPI proteins, *HWP1* and CaPir1 protein also were found to be important in hyphae formation and

formation of the cell wall. Plus, the deletion of these glycoprotein genes showed attenuate virulence in *C. albicans* (Alberti-Segui *et al*., 2004).

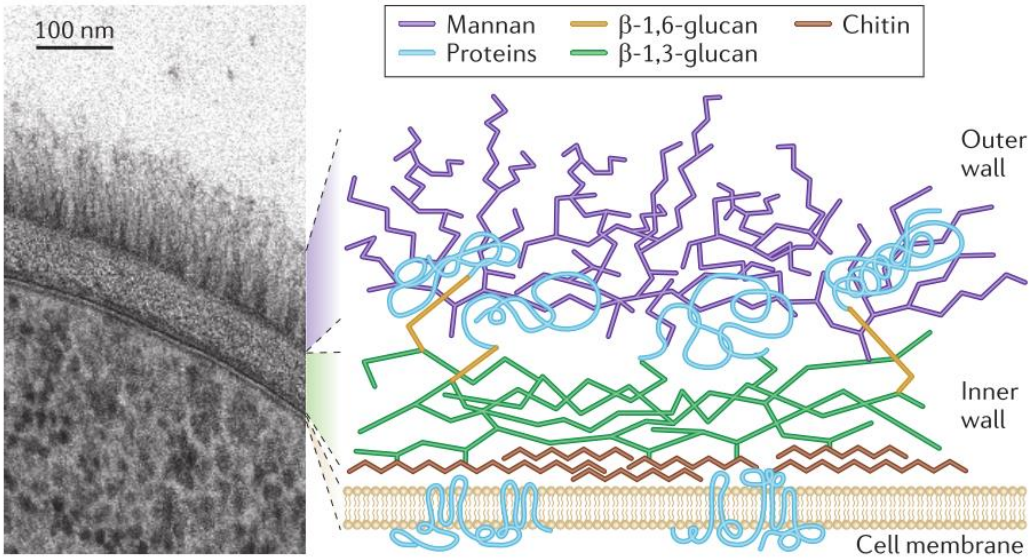

Figure 2.1 The structure of *C. albicans* cell wall. The outer layer consists of mannan and protein and associated by covalent bond forming glycoproteins. Meanwhile the inner layer consists of chitin and β-1,3-glucan. Both, inner and outer layer were linked through flexible β-1,6-glucan (adapted from Gow et al., 2011).

Furthermore, the ability of *C. albicans* to adhere to the host also contributes to its pathogenicity. Several genes were triggered by numbers of cell wall proteins including agglutinin-like (Als) family, glycosylphosphatidylnositol-anchored glucan cross linked cell wall protein (Eap1) and hyphal wall protein1 (Hwp1) (Li and Palecek, 2008; F. Li *et al*., 2007). Adherence of *C. albicans* to the host surface will promote the formation of hyphae. This is due to the ability of *C. albicans* to switch its phenotypes for further infection process.

In addition, *C. albicans* was known as polymorphic yeast thus it existed in different morphology either budding yeast, pseudohyphae or hyphae form as shown in Figure 2.2. Either yeast or hyphae both are important for virulence and plays a crucial role at different phase of infection starting from adherence and penetration to the host and its ability to escape from immune cells. Phenotypic switching in *C. albicans* involved the genes encode for hyphae development such as a transcriptional regulator (*UME6)*, hyphae G1-type cyclin 1 *(HGC1)*, hyphae wall protein (hwp1), adhesin agglutinin-like protein 3 (Als3) and Tup1 protein 5 *(RBT5)*. Plus, these proteins also were indirectly involved in biofilm formation and might increase resistance in *C. albicans* (Nobile *et al*., 2006)

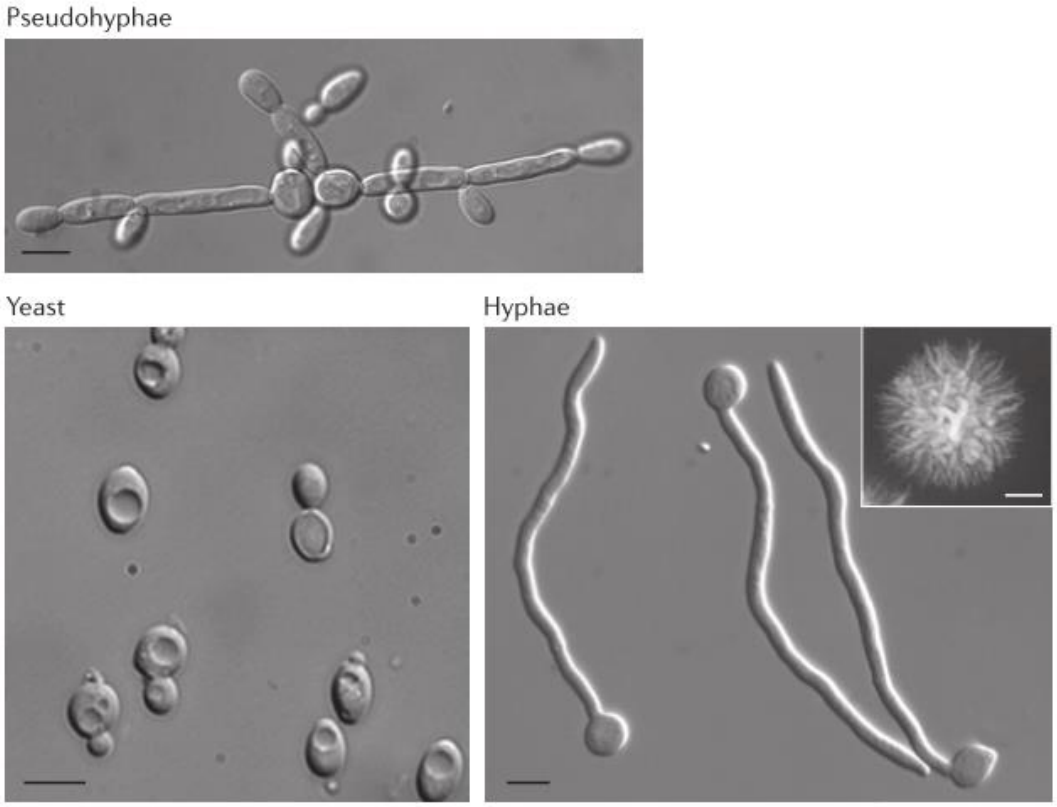

Figure 2.2. *C. albicans* morphology; yeast, pseudohyphae and hyphae (Adapted from Sudbery and Peter, 2011)

Besides, ability to hydrolase host tissue also one of the *C. albicans* virulence factor. Hydrolases are the enzymes secreted by *C. albicans* to facilitate the penetration of hyphae into the host cells. Several genes encode for hydrolase secretion in *C. albicans* are aspartyl proteases (SAPs), phospholipases (*PLB1-5*) lipases (*LIP1-10*) (Mayer and Hube, 2013) and agglutinin like sequence (*ALS*) (Nailis *et al*., 2010). For example, *PLB1-5* is highly expressed in invasive infection due to the degradation of ester linkages in glycophospholipids of host cell wall.

Meanwhile, aspartyl proteases group *SAPs 1-10* which encode for proteinase secretion was involve in degradation of host tissue in order to utilize nutrient from the host. *SAPs1-3* were expressed during yeast phase, while *SAPs 4-6* were expressed during hyphal phase. SAPs9 and *SAPs10* were expressed in both conditions. SAPs have been confirmed to have role in *C. albicans* colonisation, cell adherence and tissue penetration. Loss of these genes resulted in decreasing virulence in mouse model (Naglik *et al*.,2004). Another group of hydrolytic enzyme is lipase. *LIP1-10* genes encode for lipase secretion. In *in vitro* study, among all *LIP* genes, *LIP1*, *LIP2, LIP9* and *LIP10* were highly expressed in biofilm *in vitro* but not *in vivo* and reconstitute human epithelium (RHE) model (Nailis *et al*., 2010).

On top of that, *C. albicans* infections arose mainly due to the ability of the yeast to form biofilm and colonise the tissue surface. Additionally, the yeast biofilm also was found to colonise implanted medical devices such as dental implants, catheters, heart valves, vascular bypass graft and ocular lenses contribute to the formation of this biofilm (Bürgers *et al*., 2010; Kojic *et al*., 2004; Hawser and Douglas, 1994).