IN-VITRO BIOLOGICAL ACTIVITIES OF Au AND Ag NANOPARTICLES BIOSYNTHESIZED USING *Commelina nudiflora* L. AQUEOUS EXTRACT

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ABSTRACT

In this study, *Commelina nudiflora* L. aqueous extract was used as a reducing and stabilizing agent for the synthesis of metallic gold and silver nanoparticles. The physico-chemical and biological properties of the biosynthesized gold and silver nanoparticles were studied in a nanoscale regime. The synthesized gold and silver nanoparticles physico-chemical properties were characterized by various analytical techniques such as UV-VIS, FESEM, XRD and FT-IR. The synthesized gold and silver nanoparticles were monodispersed, and the controlled shapes and tuneable surface properties were proven. Also, the reaction parameters such as pH, temperature, plant extract concentration and metal ion concentration have been optimized to synthesize the specific sizes and shapes of the nanoparticles. The synthesized gold and silver nanoparticles were spherical and triangular in shapes with the size range of between 25 to 45 nm and 50 to 150 nm respectively. The EDX spectra show strong peaks of both gold and silver nanoparticles which are more than 80% in the sample. The XRD data supports the claim that synthesized gold and silver nanoparticles are crystalline in nature. The plant extract contains various phytochemical constituents such as saponins, alkaloids, flavonoids and phenolic compounds. These secondary metabolites may be responsible for the Au and Ag ions reduction and also help in the formation of the metal nanoparticles. Furthermore, the *in-vitro* antioxidant ability of *C. nudiflora* extracts was studied by DPPH and ABTS radical scavenging assays. The aqueous plant extract showed significant activity in the free radical scavenging which were 63.4 mg/GAE and 49.10 mg/g in DPPH and ABTS respectively. Furthermore, the biosynthesized gold and silver nanoparticles have shown reduction in the cell viability and increased cytotoxicity on HCT-116 colon cancer cells with IC_{50} concentration of 200 and 100 µg/ml. The flow cytometry experiments revealed that the gold and silver nanoparticles treated cells increased DNA fragmentation and significant changes were observed in sub G1 cell cycle phases compared with positive control. Finally, the mRNA gene expressions of HCT-116 cells were studied by RT-qPCR techniques. The pro-apoptotic genes were highly expressed in the gold nanoparticles treated HCT-116 colon cancer model. The apopototic genes such as PUMA (++), caspase-3 (+) and caspase-8 (++) were moderately expressed in the treated samples compared with cisplatin. Overall, these findings prove that the *C. nudiflora* extract successfully synthesize metallic gold and silver nanoparticles with controlled size and shapes and also acts as a potent anti-colon cancer drug in the near future.

ABSTRAK

Penyelidikan ini adalah untuk menggunakan ekstrak akues tumbuhan *Commelina nudiflora* L. sebagai penstabil serta agen penurunan bagi penghasilan partikel nano logam emas dan perak menggunakan kaedah biosintesis. *C. nudiflora* tumbuhan rumpai yang boleh dimakan, ekstraknya digunakan untuk biosintesis nanopartikel emas dan perak dan pencirian fisio-kimianya dengan pelbagai teknik analisis seperti UV-VIS, FESEM, XRD dan FT-IR. Partikel nano emas dan perak yang dihasilkan secara biosintesis perlu diciri fizik-kimia dan biologinya pada skala nano. Partikel nano emas dan perak yang disintesis memiliki ciri pembauran mono seragam, bentuk terkawal dan sifat-sifat permukaan boleh ubah. Usaha untuk mengoptimumkan parameter tindakbalas seperti pH, suhu dan kepekatan ekstrak tumbuhan dan ion kepekatan logam untuk mensintesis saiz dan bentuk partikel nano tertentu juga dijalankan. Hasilnya menunjukkan bahawa pencirian fizik-kimia dari partikel nano emas dan perak masing-masing adalah bersifat kristal dengan pelbagai saiz antara 25-45 nm dan 50-150 nm. Juga, partikel nano emas dan perak yang terhasil secara biosintesis adalah berbentuk bulat dan segi tiga dilaporkan dalam kajian ini. Spektrum EDX menunjukkan puncak tenaga isyarat yang kuat daripada kedua-dua emas dan perak atom dalam julat di antara 2-3 keV. Sebaliknya, data XRD menyokong partikel nano emas dan perak disintesis adalah dalam keadaan kristal secara semula jadi. Juga, kami telah mengenal pasti beberapa juzuk fitokimia awal seperti saponin, alkaloid, flavonoid dan sebatian fenolik daripada ekstrak tumbuhan *C. nudiflora* menggunakan pelarut berbeza polariti. Metabolit sekunder mungkin turut terlibat dalam tindak balas penurunan dan juga membantu dalam pembentukan partikel nano logam. Keupayaan anti-oksidan in vitro ekstrak *C. nudiflora* dikaji dengan penentuan DPPH dan ABTS pencarian radikal. Ekstrak tumbuhan berair menunjukkan aktiviti yang penting dalam mengaut radikal bebas daripada 63.4 mg /GAE dan 49.10 mg /g dalam DPPH dan ABTS. Partikel nano logam emas dan logam perak terhasil dari ekstrak tumbuhan *C. nudiflora* ini dengan ketara mengawal pertumbuhan HCT-116 sel-sel kanser kolon secara *in-vitro*. Logam nano partikel emas dan perak yang terhasil telah berjaya mengurangkan sel hidup dan meningkatkan kadar sitoktoksik pada sel kolon HCT-116 dengan kadar IC_{50} 200 dan 100 μg / ml. Tambahan pula, eksperimen aliran sitometri menunjukkan kadar kepekatan IC_{50} sel yang dirawat dengan partikel nano emas dan perak menunjukkan peeningkatan fragmentasi DNA dan perubahan ketara diperhatikan pada sub G1, S, G2 fasa kitaran sel berbanding dengan kawalan. Ekspresi gen mRNA dalam HCT-116 telah dikaji dengan teknik QRT-PCR. Gen apoptotik amat terekspresi dengan tinggi dalam model HCT-116 kolon kanser yang dirawat, seperti PUMA (++), dan caspase-3 (+), *caspase*-8 (++) dengan ekspresi sederhana sampel dirawat berbanding dengan *cisplatin*. Secara keseluruhan, hasil dapatan ini telah menunjukkan bahawa ekstrak *C. nudiflora* sebagai sumber baru untuk sintesis logam partikel nano emas dan perak dengan saiz dan bentuk dikawal dan juga ia boleh diguna sebagai dadah anti-kanser kolon yang potensi dalam masa terdekat.

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
AuNP	Gold nanoparticles
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
e.g	(example gratia) for example
ECM	Extra-cellular matrix
EDS	Energy-dispersive X-ray spectroscopy
et al.,	(er alia); and others
FACS	Fluorescence activated cell scanning
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
Fig	Figure
g	gram
GNPs	Gold nanoparticles

HCT-116	Human colon cancer cell line
hrs	Hours
IC_{50}	Inhibitory concentration at 50%
mg	Milligram
min	Minute
ml	Milliliter
MMP	Matrix metalloprotenase
mRNA	messenger ribonucleic acid
MTT	3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	Molecular weight
N	sample size
NaOH	Sodium hydroxide
NCI	National cancer institute
nm	nanometer
OD	optical density
p	the probability of obtaining the results
PBS	phosphate buffer saline
pH	phosphate ion concentration
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction
TEM	Transmission electron microscopy
v/v	Volume/volume

WHO	World Health Organisation

LIST OF SYMBOLS

*	Statistical significance denotation
µg	Microgram
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.0 CHAPTER OVERVIEW

This chapter describes the rationale of this research. The literature review shows that metallic nanoparticles synthesized from plant resources offer antibacterial and anticancer properties. In addition, the scope of the study presents the synthesis of metallic nanoparticles using biological route and their biomedical applications. Finally, the research objectives are also provided.

1.1 BACKGROUND OF THE STUDY

Nanoparticles (NP) are the building blocks in nanotechnology and they have diverse applications in different fields such as biomedical, engineering, energy and environmental sciences. In general, nanoparticles are synthesized by physical and chemical procedures, as these methods produce the desired sizes of the particles in large scale (Akamatsu et al., 2003, and Seigneuric et al., 2010). However, the physical methods have some limits such as expensive, involve time consuming steps and complicated vacuum techniques are necessary. Usually, the chemical processes have two main problems. Firstly, high surface energy of nanoparticles may enhance the interaction with materials and they often undergo aggregation. This aggregation can be prevented by using polymers, surfactants and DNA on the nanoparticle surface. Secondly, concentrated chemicals are used as reducing and stabilizing agents (sodium borohydride, citric acid etc.) which may exhibit biological hazards to humans and the environment (Bigall and Eychmuller, 2010, and Antony et al., 2011). Hence, the chemical and physical syntheses of nanoparticles have limited applications in the

clinical fields. Therefore, the biological synthesis of nanoparticles is the alternative by using plants and microorganisms as substrates. Moreover, the biological mediated metallic nanoparticles are proven to be more biocompatible and have lower environmental toxicity. Thus, they can be useful for different biological applications including cancer treatments. The metallic NPs have been developed by using biological methods and evaluated in various preclinical or clinical studies, some of which have been approved for clinical cancer treatments (Chow, 2010; Reza Ghorbani et al., 2011). Moreover, the biosynthesized NPs also have the ability to reduce drug resistance and enhance therapeutic applications against chronic diseases. The biosynthesized metallic gold and silver nanoparticles are feasible drugs for treating cancer effectively due to the potent physico-chemical properties.

Cancer is the third leading cause of death worldwide after coronary diseases and diabetes. According to the World Cancer Report 2008 by WHO, the global cancer burden has doubled in the last four decades of the 19th century (Chithrani et al., 2006). In Malaysia, cancer is the second most dangerous class of disease. Among cancer, the colon cancer shows the highest rate recorded in Chinese and Indian followed by Malay citizens (Lim et al., 2006). Colon cancer is one of the most dangerous class of cancer and an early detection is difficult to be made. The cancer cells in the colon or rectum divide fast and uncontrollably, ultimately forming a malignant tumor. The colon and rectum are parts of the digestive system, which take up nutrients from food and water in the colon. Colon cancer is common in both men and women. The preliminary colon polyps can develop into malignant tumors (Jain et al., 2007). The traditional strategies for cancer treatment are surgery, radiation, and chemotherapy. But, these specialized therapies can be applied only at the preliminary stage of cancers. The physical method of cancer treatment is surgery. It is a good way to cure, particularly those which have yet to metastasize to distinct parts of the body (Douglas-Kinghorn, 2001). Once it is metastasized, the multiplications of cancer cells are difficult to be controlled. Therefore, these stages need new and more effective therapies.

The nanoscience has proposed many fabrication methodologies including biological synthesis method. The biological synthesis method has developed unique and

precise nanoparticles and it is possible to target cancer at different stages. On the other hand, the chemically synthesized nanomaterials also have specific sizes and shapes, but they are futile in clinical trials because of toxicity issues (Yoosaf et al., 2007). Therefore, the biosynthesis way is more effective, safe and may fulfill the following requirements: i) the drug concentration can be easily optimized which allows an effective dose at tumor cells without affecting normal cells, ii) could target tumor cells and prevent an uptake by normal cells, and iii) biological approach has a high biocompatibility.

Nanoparticle is defined as a sub-microscopic particle with the size that ranges between 1 to 100 nm. When the size of materials is reduced to the nano level, the properties change completely compared to bulk materials (Canizal, 2001, and Chaloupka et al., 2010). Gold nanoparticle (GNP) is a novel metal, has been utilized in many areas especially cancer diagnostics, coatings, thermal therapy, electronics and biotechnology (Gardea-Torresdey et al., 2003, and Kumar et al., 2007). GNPs can easilypass through the vasculature, be localized in targeted areas, and control the DNA transcription in cancer cells. The biological syntheses of gold nanoparticles are cheap, reliable and eco-friendly because of the naturally available plants acting as reducing and stabilizing agents and do not require any downstream process for purification of products. The plant extract contains various bioactive compounds which is able to reduce metal ions into metallic nanoparticles at room temperature (Sau et al., 2010).

On the other hand, silver nanoparticles also have unusual properties such as high antimicrobial activity, particle stability and surface chemistry (Krug et al., 1999, and Labouta and Schneider, 2010). Silver nanoparticles have specific surface plasmon resonance (SPR) peak wavelengths of between 450 nm (violet light) to 530 nm (green light). Different wavelengths express different particle sizes, shapes and surface properties (Jain et al., 2007). The AgNPs have been widely used as antimicrobial agents in healthcare, food industry, textile coatings and electronic devices (Reza-Ghorbani et al., 2011). Also, the AgNPs have been incorporated in many commercial products and approved by a range of accredited bodies, including the FDA (USA), SIAA (Japan) and KTR and FITI (Korea) (El-Nour et al., 2010).

1.2 PROBLEM STATEMENT

In this generation, the nanosized materials are very popular and they have numerous applications in different fields. The synthesis of nanoparticles by chemical and physical methods have been well established and successfully reported in literature. But, these methods use chemical precursors as a stabilizing and capping agents to promote the synthesis reaction. Therefore, these syntheses are not suitable for clinical use and harmful for living organisms and higher animals. However, plant extracts could act as a natural reducing and capping agent in reducing the reaction. The wide availability of bioactive compounds (metabolites) guarantees the metal ions reduction into metallic nanoparticles. In this study, potential edible weed plant *C. nudiflora* aqueous extract was used for the nanoparticles synthesis by environmental approaches.

On a different note, the increasing mortality of colon cancer cases in Asian countries is a big problem that needs to be controlled and treated. Also, the available cancer drugs are expensive and ineffective at different stages of cancer. According to the report of National Cancer Registry (Malaysia) the most frequent cancer cases is breast cancer and followed by colon cancer. The synthetic anticancer drugs (cisplatin, doxorubicin etc.,) arecostly and involve multiple purification processes in developing the product. The biosynthesis of metallic nanoparticles using plants is becoming a more fashionable and promising in drug development. However, other biolological resources such as bacteria, fungi and algae need a huge investment for a large scale culturing and maintancence. Due to that, plant resources could be a better alternative resource for nanoparticles synthesis. The biosynthesis of nanoparticles uses plant extracts with no addition of any chemical stabilizing and capping agents, therefore, it could be 99% useful for all clinical studies. The plant mediated nanoparticles are highly effective, cost less and could be a counter point for future cancer therapy. In this study, *C. nudiflora* plant extract was used to synthesize gold and silver nanoparticles and evaluate the potent anticancer properties in HCT-116 colon cancer cells. Nevertheless, the mechanism of nanoparticle formation and function of colon cancer activity need to be explored in future. Although, the different molecular assays support the potential of

synthesized gold and silver have potent for *in-vitro* anticancer activities, the animal model studies are needed for further confirmation of metallic nanoparticle functions and their behavior.

1.3 RESEARCH OBJECTIVES

In this PhD work three objectives are focused. The specific goals were as follows:

- To biosynthesize Au and Ag nanoparticles using *Commelina nudiflora* aqueous extract and to characterize them by different analytical techniques such as UV-Vis, FESEM, EDX, XRD, FTIR, particle size analyser and zeta potential.

- To isolate and conduct a preliminary identification of the potential bioactive compounds from *C. nudiflora* extract using GC-MS.

- To evaluate *in-vitro* anticancer efficacy of synthesized Au, Ag nanoparticles against HCT-116 colon cancer cells and to conduct molecular characterization of HCT-116 colon cancer cells using flow cytometry and RT-qPCR techniques.

1.4 SCOPE OF THE STUDY

The goal of this study is to develop metallic Au and Ag nanoparticles using *C.nudiflora* aqueous extract and to study their potential biomedical applications such as *in-vitro* antibacterial and anticancer activities.The following research objectives require different experiments. For the first objective, the following experiments were used:

- Identify the weed plant, *C.nudiflora* for the biosynthesis of Au and Ag nanoparticles
- Optimize the ratio of plant extract (10 ml, 25 ml, 50 ml and 100 ml) and metal ion (10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M) solution

- Optimize the temperature (35 °C, 50 °C, 60 °C, 70 °C) and pH (6, 7, 8) of the mixture of plant extract and metal precursor solution
- Identify the morphology, crystalline nature and metal composition using FESEM, XRD and EDX ,then, the functional groups and study the thermal stability by using FT-IR, TGA
- Identify the antibacterial and antioxidant properties of synthesized Au and Ag nanoparticles

For the second objective, the following procedures were used,

- Isolate bioactive metabolites from *C.nudiflora* using soxhlet apparatus
- Identify the phytochemical constituents using standard phytochemical screening procedure and GC-MS techniques
- Identify the antibacterial and antioxidant activity of *C.nudiflora* plant extract

For the third objective, the following expeiments were carried out,

- Measure the cytotoxicity of Au and AgNPs against HCT-116 colon cancer cells
- Study the cell cycle phases in control and Au, Ag nanoparticles treated HCT-116 cells
- Determine the apoptotic genes expression in colon cancer cells treated with Au and Ag nanoparticles and cisplatin

1.5 STATEMENT OF THE CONTRIBUTION

This study produces biosynthesized Au and Ag nanoparticles using *Commelina nudiflora* aqueous extract for the first time. Thus, the *C.nudiflora* synthesized metal nanoparticles have the potential for *in-vitro* and the antibacterial antioxidant properties are proven. It is also reported that the *C.nudiflora* aqueous extract contains a cluster of bioactive metabolites which acta as a natural reducing and stabilizing agent. Finally, it is reported for the first time that the biosynthesized Au and Ag nanoparticles h effectively treated the HCT-116 colon cancer in *in-vitro*.

CHAPTER 2

REVIEW OF LITERATURE

2.0 CHAPTER OVERVIEW

This chapter describes the literature related to this research, various methods of synthesis of metallic nanoparticles using bio-substrates and their biomedical applications. Besides, different methods used to synthesize metallic nanoparticles and their bioactivities such as antimicrobial, antidiabetic and anticancer properties are also shown. In addition, the pros and cons of nanoparticles synthesis using other synthesis methods such as physical and chemical methods are also discussed. The detailed studies on plants used for the synthesis of metallic nanoparticles and its redox mechanisms are included. Furthermore, a few cancer molecular techniques and their principles are discussed too.

2.1 NANOMEDICINE

Nanoparticles promise a revolutionary in modern medicine for diagnosing and treating various chronic diseases particularly cancer and microbial infections (Dreaden et al., 2011). The studies in metallic nanoparticles have evolved as a major research direction in modern medicine to miniaturize drug size at nanoscale from macro scale level (Dykman and Khlebtsov, 2011). It is a well admitted science research that has the importance in several areas including medicine, pharmaceutical, opto-electronics, sensing and catalysis (Dong et al., 2007). The syntheses of monometallic and bimetallic nanoparticles such as Au, Ag and Au-Ag have shown a good impact in biomedical applications (Klabunde and Mulukutla, 2001). The biosynthesis of nanosized elements has used different resources such as plants, bacteria, fungi, micro and macroalgae (Seeman, 1982, and Liu, 2006).