GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM WATER-SOLUBLE POLYSACCHARIDES OF *CLINACANTHUS NUTANS* LEAF: EXTRACTION, CHARACTERISATION, OPTIMISATION AND ANTIBACTERIAL STUDIES

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by

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LIST OF SYMBOLS

°	Degree
°C	Degree Celsius
=	Equals
γ	Gamma
≥	Greater-than or equal to
λ	Lambda
≤	Less-than or equal to
μ	Micro
%	Percentage
−	Minus
+	Plus
±	Plus-minus

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACD_AgNP	ACD_CNP synthesised silver nanoparticles
ACD_CNP	Acid extraction of *C. nutans* leaf crude polysaccharide
Ag	Silver
Ag^+	Silver ion
$AgNO_3$	Silver nitrate
AgNP	Silver nanoparticles
ALK_AgNP	ALK_CNP synthesised silver nanoparticles
ALK_CNP	Alkaline extraction of *C. nutans* leaf crude polysaccharide
ANOVA	Analysis of variance
ATCC	American Type Cell Culture Collection
ATR-FTIR	Attenuated total reflectance (ATR)-Fourier transform infrared spectroscopy
BBD	Box-Behnken design
C. nutans	*Clinacanthus nutans*
CLSI	Clinical and Laboratory Standards Institute
CNP	*C. nutans* leaf crude polysaccharide
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
E. coli	*Escherichia coli*
EDX	Energy dispersive X-ray analysis
FT_CNP	Freeze-thaw extraction of *C. nutans* leaf crude polysaccharide
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry

h	Hour
HW_CNP	Hot water extraction of *C. nutans* leaf crude polysaccharide
IC_{50}	Half-maximal inhibitory concentration
JCPDS	Joint Committee on Powder Diffraction Standard
LC_{50}	Lethal concentration 50
M	Molar
MBC	Minimum bactericidal concentration
mg/mL	Milligram per milliliter
MIC	Minimum inhibitory concentration
min	Minute
mm	Millimeter
mM	Milli mol
MOA	Mechanism of action
mRNA	Messenger RNA
MS	Mass spectrometry
MVDA	Multivariate data analysis
nm	Nanometer
PCA	Principal component analysis
PC-DFA	Principal components-discriminant function analysis
PCR	Polymerase chain reaction
PDI	Polydispersity index
pH	Potential of hydrogen
PLS	Partial least squares
R^2	Correlation coefficient
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSM	Response surface method

s	Second
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
Standard_AgNP	Silver nanoparticles from Sigma-Aldrich
TEM	Transmission electron microscopy
TPC	Total phenolic content
UL_AgNP	UL_CNP synthesised silver nanoparticles
UL_CNP	Ultrasound-assisted extraction of *C. nutans* leaf crude polysaccharide
UV-Vis	Ultraviolet-visible spectroscopy
VIP	Variable importance in projection
XRD	X-ray diffractometry analysis
ZOI	Zone of inhibition
µg/mL	Micro gram per milliliter
µL	Micro liter

LIST OF APPENDICES

SINTESIS HIJAU NANOPARTIKEL PERAK DARI POLISAKARIDA LARUT DALAM AIR DARI DAUN *CLINACANTHUS NUTANS*: PENGEKSTRAKAN, PERINCIAN, PENGOPTIMUMAN DAN KAJIAN ANTIBAKTERIA

ABSTRAK

C. nutans telah mendapat banyak perhatian oleh penyelidik kerana sifat farmakoterapeutiknya. Walau bagaimanapun, kaedah pengekstrakan polisakarida mentah *C. nutans* (CNP), sintesis nanopartikel perak (AgNP) daripada CNP dan sifat antibakteria serta, sebagai perencat sintesis DNA belum dikaji. Oleh itu, penyelidikan ini bertujuan untuk menggunakan kaedah hijau (CNP) untuk menghasilkan AgNP dan menilai potensi penggunaannya sebagai agen antibakteria. CNP diekstrak menggunakan lima kaedah iaitu air panas (HW_CNP), ultrabunyi (UL_CNP), beku-cair (FT_CNP), alkali (ALK_CNP) dan asid (ACD_CNP). Kelima-lima CNP telah dioptimakan, dicirikan dan komposisinya telah dianalisa. Tiga CNP kemudiannya dipilih untuk mensintesis ACD_AgNP, ALK_AgNP, dan UL_AgNP masing-masing, di mana ALK_AgNP dan UL_AgNP dipilih berdasarkan keadaan sintesisnya dan ketoksikannya dalam udang pekasin dikuantitasi. Di sini, potensi sebagai agen antibakteria serta mekanisme tindakan perencatan sintesis DNA antibakteria ALK_AgNP dikaji. Keputusan CNP menunjukkan bahawa ACD_CNP dan FT_CNP memperoleh hasil pengekstrakan tertinggi dan terendah masing-masing pada 14.85 ± 0.25% dan 4.16 ± 0.13%. Manakala kandungan karbohidrat dan protein tertinggi dilaporkan oleh HW_CNP [287.72 ± 16.14 setara glukosa (µg/mL)] dan UL_CNP [19.09 ± 0.95 setara albumin (µg/mL)]. FT_CNP mempamerkan kandungan fenolik tertinggi [418.49 ± 1.91 setara asid galik (µg/mL)] dengan IC_{50} keantioksidan terendah

pada 380.37 ± 53.20 µg/mL (DPPH) dan 186.43 ± 2.93 µg/mL (ABTS). Analisis ATR-FTIR dan GC-MS mengesahkan pengekstrakan CNP, manakala metabolomik menggambarkan pemisahan CNP dalam tiga kelompok. Dari sini, keadaan sintesis optimum ACD_AgNP, ALK_AgNP, dan UL_AgNP berbeza-beza, namun; ALK_AgNP mempunyai saiz hidrodinamik terendah (66.74 ± 1.43 nm) dan kestabilan koloid tertinggi (-30.63 ± 0.86 mV). UV-Vis dan ATR-FTIR mengesahkan pembentukan AgNP masing-masing. SEM memperlihatkan struktur sfera, manakala TEM mengesahkan saiz AgNP tersebut (ALK_AgNP pada 7.48 ± 2.88 nm dan UL_AgNP pada 5.21 ± 1.92 nm). Dalam cerakin udang pekasin, peratus kematian akibat ALK_AgNP adalah lebih rendah daripada UL_AgNP selepas 24 jam. Dalam *E. coli* dan *S. aureus* pula, ALK_AgNP membentuk zon perencatan sebanyak 11.33 ± 0.58 dan 12.00 ± 1.00 mm, masing-masing. MIC dan MBC ALK_AgNP adalah dua-kali ganda lebih rendah dalam *E. coli* berbanding *S. aureus*. ALK_AgNP juga memaparkan perencatan sintesis DNA dalam kedua-dua *E. coli* dan *S. aureus*, manakala Standard_AgNP hanya memaparkanya di *S. aureus*. Oleh itu, ALK_CNP yang diekstrak dalam penyelidikan ini terbukti sebagai agen pengurangan yang berjaya untuk mensintesis ALK_AgNP dengan saiz yang kecil dan telah menunjukkan ketoksikan yang rendah dalam in vivo, serta ciri yang menjanjikan sebagai agen antibakteria yang berkesan dengan sifat perencatan sintesis DNA bakteria.

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ABSTRACT

C. nutans has gained much attention by researchers due to its pharmacotherapeutic properties. However, the extraction method of *C. nutans* leaf crude polysaccharide (CNP), CNP synthesising silver nanoparticles (AgNP) and the subsequent antibacterial properties as a DNA synthesis inhibitor, has not been studied. Hence, this research aims to employ a green synthetic method to produce AgNP and assess its potential application as an antibacterial agent. Five extraction methods of CNP were employed, namely hot water (HW_CNP), ultrasound-assisted (UL_CNP), freeze-thaw (FT_CNP), alkaline (ALK_CNP) and acid (ACD_CNP). All five CNP were optimised and characterised, and their compositions were analysed. Three CNP were then selected to synthesise ACD_AgNP, ALK_AgNP, and UL_AgNP, respectively, where ALK_AgNP and UL_AgNP were optimally selected based on their synthesis conditions and their toxicity in brine shrimp was quantified. The antibacterial and potential DNA synthesis inhibition mechanism of action (MOA) of ALK_AgNP was further quantified. The results of CNP showed that ACD_CNP and FT_CNP obtained the highest and lowest extraction yield at 14.85 ± 0.25% and 4.16 ± 0.13%, respectively. While the highest carbohydrate and protein content were reported by HW_CNP [287.72 ± 16.14 glucose equivalent (µg/mL)] and UL_CNP [19.09 ± 0.95 albumin equivalent (µg/mL)], respectively. FT_CNP exhibited the highest phenolic content [418.49 ± 1.91 gallic acid equivalent (µg/mL)] and the

antioxidant IC_{50} at 380.37 ± 53.20 µg/mL (DPPH) and 186.43 ± 2.93 µg/mL (ABTS). The ATR-FTIR functional groups and GC-MS metabolites confirmed the successful extraction of CNP, while metabolomics illustrated the discrimination in three clusters. From here, the optimisation of the synthesis condition of ACD_AgNP, ALK_AgNP, and UL_AgNP varied. ALK_AgNP reported the smallest hydrodynamic size (66.74 ± 1.43 nm) and highest colloidal stability (-30.63 ± 0.86 mV). The UV-Vis and ATR-FTIR confirmed the formation of the AgNP. The SEM revealed spherical structures, while TEM confirmed the size of AgNP (ALK_AgNP at 7.48 ± 2.88 nm and UL_AgNP at 5.21 ± 1.92 nm). In the brine shrimp assay, ALK_AgNP reported a much lower mortality percentage at 24 h compared to UL_AgNP. In *E. coli* and *S. aureus*, ALK_AgNP formed inhibition zones of 11.33 ± 0.58 and 12.00 ± 1.00 mm, respectively. The MIC and MBC were two-fold lower in *E. coli* compared to *S. aureus*. ALK_AgNP exhibited DNA synthesis inhibition in both *E. coli* and *S. aureus*, while Standard_AgNP only in *S. aureus*. Thus, the ALK_CNP extracted in this research proved to be a successful reducing agent to synthesise ALK_AgNP with a small size and had demonstrated low toxicity in vivo, as well as promising features as an effective antibacterial agent with bacterial DNA synthesis inhibition properties.

CHAPTER 1

INTRODUCTION

This chapter introduces the current research topic in a general manner and is divided into six sections. Section 1.1 provides the research background, followed by Section 1.2, which addresses the problem statements. Sections 1.3 and 1.4 address the research hypothesis and research questions, respectively. While Section 1.5 states the research aim and objectives. Section 1.6 provides information on the significance of this study.

1.1 Research background

Clinacanthus nutans (*C. nutans*) is a perennial plant found in various ASEAN countries, such as Thailand, Indonesia, Malaysia, and Vietnam. It is known locally as Belalai Gajah in Malay and Sabah Snake Grass in English (Arullappan et al., 2014; Teoh, 2021). This plant has been used as a folk medicine in Southeast Asia for many years and has gained widespread attention by researchers due to its medicinal properties. Aside from helping treat various diseases, its natural phytochemical constituents can also help maintain a healthy lifestyle (Teoh, 2021). Crude polysaccharides are polysaccharides which contain small/negligible amounts of protein and phenolic compounds (Ross et al., 2015). Naturally, polysaccharides rarely exist on their own but rather exist as a conjugate with other components which act as a whole in isolation (Chen et al., 2008). A study on tea leaf polysaccharides suggested that in the presence of polyphenols, the reducing ability and DPPH radical scavenging ability were enhanced. The findings suggested that the polyphenols triggered a synergistic elevation in the antioxidant activity (Wang et al., 2013).

Metallic nanoparticles, especially silver nanoparticles (AgNP), have gained much attention as they possess multiple pharmacological activities, including antibacterial, antifungal, anticancer, anti-inflammatory and wound healing properties (Asghar et al., 2018; Wongpreecha et al., 2018; Jacob et al., 2019). Compared to the conventional AgNP, biological/green synthesised AgNP has higher stability, better solubility, and higher yielding properties (Gurunathan et al., 2015). The crude polysaccharide method of green synthesis for AgNP is a method where AgNP are synthesised in a solution of crude polysaccharides and silver nitrate ($AgNO_3$). Aside from reducing the metal salts, the crude polysaccharide can also act as an in-situ stabilising and capping agent. The capping ability can also reduce toxicity and prevent the aggregation of the AgNP (Roy et al., 2019). Besides, there is no addition of any other chemicals, and crude polysaccharides are completely safe (Nag et al., 2022; Nkhabindze et al., 2022).

The use of microwave-assisted methods for the production of green nanocomposites and nanoparticles has been regarded as a promising green strategy (Kumar et al., 2020). The advantages of using microwaves for the production of green nanomaterials include; reduced energy consumption and shorter reaction times (Torras & Roig, 2020). Moreover, the microwave-assisted method is known to heat the mixture homogenously, allowing for the reliable nucleation and uniform size distribution of nanoparticles (Kumar et al., 2020). Microwave heating is a better method for producing smaller sizes of nanostructures due to its higher degree of crystallisation, keeping the agglomeration of the particles in check (Torras & Roig, 2020). Besides, the synthesis parameters can be altered to acquire the desired nanoparticle properties; for instance, the concentration of certain solvents, precursors,

and temperature can be used to control the size and morphologies of metallic nanoparticles (Kumar et al., 2021).

Silver has been shown to possess antimicrobial properties (Bindhu et al., 2014; Manikandan et al., 2015). Even though the exact mechanism of AgNP antibacterial effects has not been completely explained, several mechanisms have been proposed. For instance, releasing silver ions from AgNP can be considered as the mechanism of killing microbes (Bapat et al., 2018). Silver ions can adhere to the cell wall and cytoplasmic membrane owing to electrostatic attraction and affinity to sulphur proteins, and this leads to the disruption of the bacterial envelope (Khorrami et al., 2018). After the uptake of free silver ions into cells, respiratory enzymes can be deactivated by interrupting adenosine triphosphate production, and reactive oxygen species (ROS) will be generated (Ramkumar et al., 2017). ROS can be a principal agent in the provocation of cell membrane disruption and deoxyribonucleic acid (DNA) modification. Moreover, silver ions can inhibit protein synthesis by denaturing ribosomes in the cytoplasm (Durán et al., 2016).

Antibiotics are drugs that are used to treat bacterial infections. They can inhibit growth or kill bacteria (Kourkouta & Plati, 2018). Antibiotics are categorised based on their nature and their chemical structure or based on their mechanism of action. The 4 main groups of antibiotics based on their mechanism of action are: cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors, and metabolic activity inhibitors (Verma et al., 2022). Fluoroquinolones are a class of antibiotics known to inhibit two enzymes (DNA topoisomerases) involved in bacterial DNA synthesis. These enzymes are required for the replication of bacterial DNA, while in human cells, these enzymes function as homodimers. Due to this difference, the clinically-relevant quinolones can discriminate between the human and bacterial type

II topoisomerases (Aldred et al., 2014; Naeem et al., 2016). This enables fluoroquinolones to be both bactericidal and target-specific (Hooper & Jacoby, 2016).

The basic mechanism of action is that the quinolones interact with two enzymes, namely topoisomerase II and topoisomerase IV, in the bacterial cell. Topoisomerase II expresses the DNA gyrase gene, which is composed of g*yrA* and g*yrB* subunits, while topoisomerase IV is composed of two subunits as well, which are *parC* and *parE*. There are another two enzymes, namely topoisomerase I and III, which are also present in bacteria cells but are not the direct targets of quinolones. Nevertheless, topoisomerase I (encodes *topA* gene) does have a role that complements topoisomerase II, which resists the activity of DNA gyrase by preventing and removing negative supercoils from circular DNA (Sutormin et al., 2019; Villain et al., 2021). While topoisomerase III (encodes *topB* gene) is known to play a key role in the stabilisation of the bacterial genome (Yan et al., 2019).

To evaluate the association of all the factors listed above, in this study, five extraction methods of *C. nutans* leaf crude polysaccharide (CNP) was optimised and characterised in terms of their physicochemical properties and then quantified. Three CNP were selected to synthesise AgNP, which was optimised by response surface method (RSM) to obtain the smallest nanoparticle size, with the highest conjugation efficiency. The toxicity of the AgNP was evaluated (in vivo), and the safest CNP-synthesised AgNP was chosen for testing of the antibacterial activity and antibacterial DNA synthesis mechanism. The outcomes of this project would provide knowledge and evidence for the association of crude polysaccharide extraction methods and their effect on the synthesised AgNP properties. This could potentially provide new tools for designing new antibacterial agents as a safer alternative treatment that would promote human health and well-being.

1.2 Problem statements

Over the years, *C. nutans* has attracted widespread attention due to its proven medicinal properties in treating snake bites, viral infections, inflammatory diseases as well as some cancers (Kamarudin et al., 2017; Chia et al., 2021; Mohamed et al., 2022). However, the water-soluble crude polysaccharides from this plant have not been studied. Crude polysaccharides have recently gained interest due to their notable antioxidant activity (Cheng & Huang, 2018; Hamed et al., 2020; Huo et al., 2022). Besides, different extraction methods would possibly extract different bioactive crude polysaccharides. Therefore, this study evaluated the compositions of the various extracts of crude polysaccharides from the *C. nutans* leaf.

Apart from that, the understanding of the formation/synthesis of silver nanoparticles (AgNP) utilising crude polysaccharides as a reducing, stabilising and capping agent is still limited. This green synthesis of AgNP using crude plant polysaccharides has numerous advantages, including cost-effectiveness, environmentally-friendly and non-toxic (Nag et al., 2022; Nkhabindze et al., 2022). However, the green synthesised method used for AgNP is also an essential aspect of increasing the pharmacological effect. Therefore, a more recent technique of synthesis, utilising the microwave convection method, was employed to obtain uniform-size distributed AgNP with reduced energy consumption and shorter reaction times (Anjana et al., 2021; Kumar et al., 2020). Thus, this research optimised the synthesis of AgNP from *C. nutans* leaf crude polysaccharides (CNP) via the microwave-assisted method.

Moreover, commercially available and green-synthesised AgNP has always been regarded as a potent antibacterial agent against both gram-positive and gram-negative bacteria (Wongpreecha et al., 2018; Roberto Vazquez-Muñoz et al., 2020;

Urnukhsaikhan et al., 2021). However, until today, there is still a lack of research investigating the antibacterial mechanism of action (MOA) of AgNP. So far, research has identified that AgNP attaches and penetrates the bacterial cell wall, leading to silver ion leakage into the cytosol and causing cell death (Xu et al., 2019). Another possible mechanism proposed was that the influx of AgNP possibly causes an increase in oxidative stress, leading to bacterial protein and DNA damage; and the disruption of the signal transduction pathways in bacteria, potentially inducing bacterial cell apoptosis (Durán et al., 2016; M. Chen et al., 2019). Therefore, this research aimed to investigate the MOA of the CNP-synthesised AgNP by focusing on the bacterial DNA synthesis inhibition by quantifying the mRNA expression of *gyrA*, *gyrB*, *parC*, *parE*, *topA*, and *topB* genes.

1.3 Research hypothesis

i. Different extraction methods of CNP would produce extracts that possess different characteristic features.

ii. The selected CNP could act as reducing, stabilising and capping agents to synthesise/optimise AgNP.

iii. The selected CNP synthesised AgNP could be non-toxic against brine shrimp.

iv. The selected CNP synthesised AgNP may exhibit antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

v. The antibacterial mechanism exhibited by the selected CNP synthesised AgNP against *E. coli* and *S. aureus* could be by inhibiting the synthesis of bacterial DNA.

1.4 Research questions

i. Do CNP produced from different extraction methods possess different characteristic features?

ii. Could the selected CNP acts as reducing, stabilising and capping agents to synthesise/optimise AgNP?

iii. What is the toxicity level of the selected CNP-synthesised AgNP in brine shrimp?

iv. Does the selected CNP-synthesised AgNP have antibacterial activity against *E. coli* and *S. aureus*?

v. Does the selected CNP-synthesised AgNP act as a DNA synthesis inhibitor against *E. coli* and *S. aureus*?

1.5 Research aim and objectives

This research aims to employ a green method for the production of AgNP using CNP and assess the potential application of this nanoparticle as an antibacterial agent.

1.5.1 Objectives

i. To extract, optimise, characterise and evaluate the composition of five different CNP obtained from different extraction methods.

ii. To synthesise, optimise and characterise AgNP derived from three selected CNP using the microwave-assisted method.

iii. To determine the in vivo toxicity exhibited by the selected CNP synthesised AgNP using brine shrimp assay.

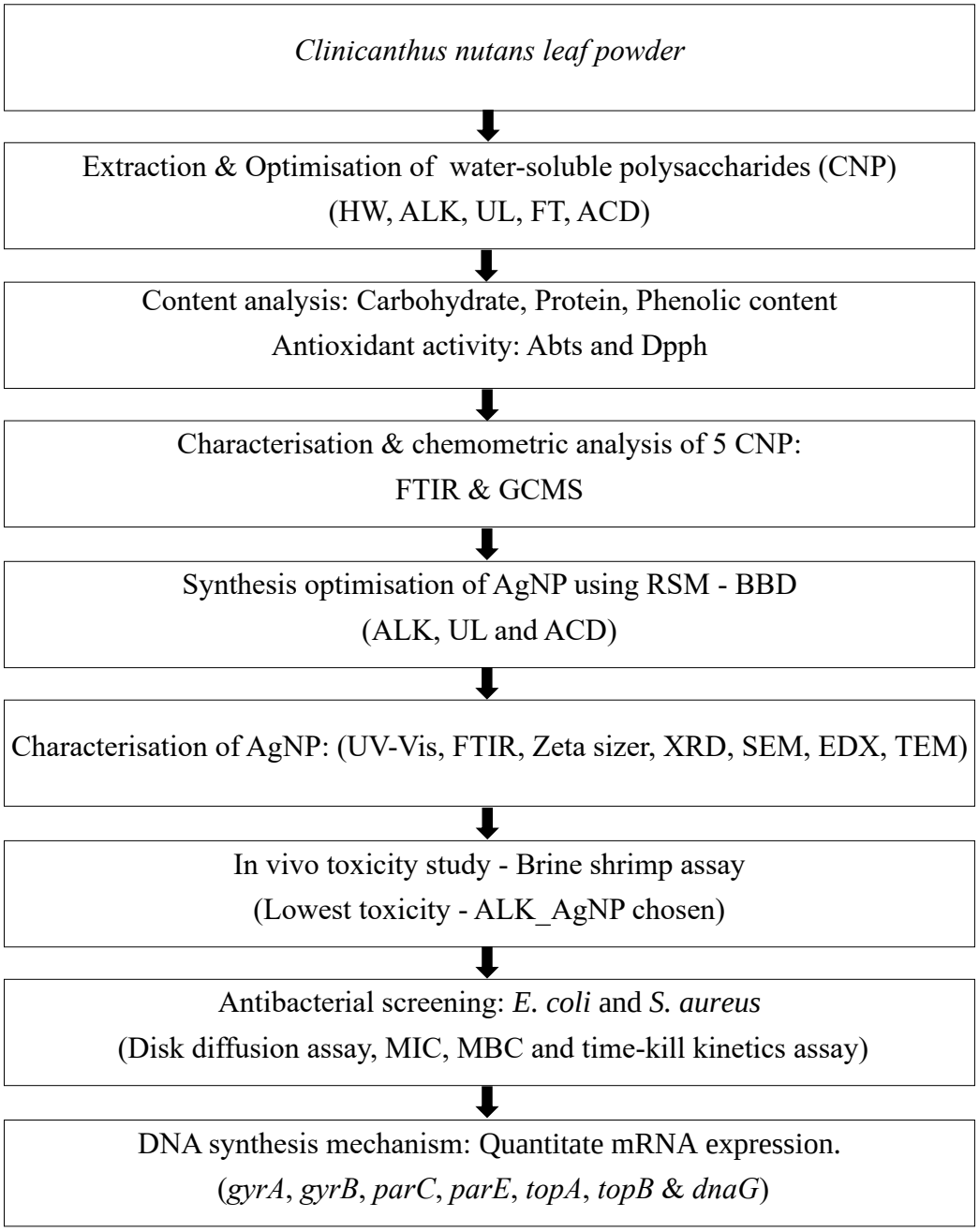
iv. To investigate the antibacterial activity exhibited by the selected CNP synthesised AgNP against *E. coli* and *S. aureus.*

v. To investigate the bacterial DNA synthesis mechanism by quantifying the targeted gene mRNA expression upon treatment with the selected CNP synthesised AgNP in *E. coli* and *S. aureus.*

1.6 Significance of the study

This project will provide knowledge on the different extraction methods of CNP and their characteristics, as well as the relationship between the CNP-synthesised AgNP physicochemical properties and their toxicity, enabling the production of more efficient AgNP compared to chemically synthesised nanoparticles. Besides, this study also elucidates a novel antibacterial mechanism of the selected CNP synthesised AgNP in relation to the bacterial DNA synthesis genes. This may contribute to the development of a new antimicrobial product utilising plant-mediated green synthesis.

1.7 Flow chart of the study



CHAPTER 2

LITERATURE REVIEW

This chapter describes the literature review conducted to gain an understanding of the existing research and debates relevant to this research project. Section 2.1 summarises the *Clinacanthus nutans* (*C. nutans*) phytochemical compounds and pharmacological activities. Section 2.2 details about carbohydrates and their antioxidant activity. Meanwhile, Section 2.3 reviews the definition and uses of metabolomics; while Section 2.4 reviews silver nanoparticles, their synthesis mechanism, characterisation techniques and their antibacterial mechanism of action. Section 2.5 reviews the brine shrimp assay, while Section 2.6 details the general topic of gram-positive and gram-negative bacteria. Next, Section 2.7 reviews the classes of antibiotics, specifically the fluoroquinolone class of antibiotics and Section 2.8 reviews the univariate and multivariate data analyses used in this research.

2.1 *Clinacanthus nutans (C. nutans)*

Clinacanthus nutans is a perennial plant that can be found in various Southeast Asian countries, such as Thailand, Indonesia, Malaysia, and Vietnam. It is locally known as Belalai Gajah in Malay and Sabah Snake Grass in English (Figure 2.1) (Arullappan et al., 2014; Teoh, 2021). In Thailand and Malaysia, it is believed that *C. nutans* can cure snake bites, viral infections, and some cancers, including breast, colon, stomach, lung, and liver cancer (Kunsorn et al., 2013; Kamarudin et al., 2017).

C. nutans has also been used to treat various illness such as fever, diabetes, and gastrointestinal diseases (Shim et al., 2013). Its fresh leaves can be used alone or with other herbs for general well-being (Aslam et al., 2015). In Thailand, the leaves were commonly applied to treat insect bites, skin irritations and lesions caused by certain

viruses (Varicella zoster virus and Herpes simplex virus) (Sakdarat et al., 2019). Its phenolic compounds have antioxidant properties that can help cancer patients (Teoh, 2021). In Malaysia, some cancer patients claimed that they were able to recover after drinking a decoction made with *C. nutans* leaves; however, there is a lack of scientific studies and evidence to support this claim. Aside from helping treat various diseases, its natural phytochemical constituents can also help maintain a healthy lifestyle (Teoh, 2021).

Figure 2.1 *Clinacanthus nutans* plant. Plant Source: Herb Garden, Animal Research Complex, Advanced Medical and Dental Institute, Universiti Sains Malaysia.

2.1.1 *C. nutans* phytochemical compounds

Various phytochemicals were identified from various solvent extracts of *C. nutans* plant. These include lupeol, stigmasterol, and myricyl alcohol (Arullappan et al., 2014; Teoh et al., 2017). From the butanol and water extracts of *C. nutans*, six C-glycosyl flavones, which are isomollupentin 7-O-β-glucopyranoside, isoorientin,

isovitexin, orientin, schaftoside and vitexin were isolated (Tuntiwachwuttikul et al., 2004).

Satakhun et al. (2001) reported the discovery of two glycoglycerolipid compounds found in the leaves. These compounds are 1-O-palmitoyl-2-Olinolenoyl-3-O-D-galactopyranosyl-glycerol and 2-O-lipoyl-1-O-glycerol. A total of nine compounds, including a monoacylglycerol, were isolated from the leaf ethyl acetate fraction (Tuntiwachwuttikul et al., 2004). An antimicrobial compound known as 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester was also isolated from the chloroform leaf extract (Yong et al., 2013). Aside from having phenolic acids (caffeic acid and gallic acid), *C. nutans* extracts also have flavonoids, such as catechin and kaempferol (Ghasemzadeh et al., 2014).

Moreover, *C. nutans* contains various phytochemicals which have anti-inflammatory and antioxidant properties. Some of these include lupeol, catechin, and vitamin E. Studies on various bioactive compounds have shown that they could treat conditions such as hyperuricemia and wound healing. Among the compounds studied were isovitexin, cycloclinacoside-A1, and shaftoside (Radhakrishnan et al., 2016).

2.1.2 *C. nutans* pharmacological activities

The various extraction solvents used to extract *C. nutans* and their reported pharmacological activities are as summarised in Table 2.1.

Table 2.1 The pharmacological activities of *C. nutans* leaf extracts.

Extraction solvent	**Pharmacological activities**	**Reference**
Acetone	Antimicrobial properties against several pathogenic gram-positive, gram-negative strains and fungi strains.	(Kong & Abdullah Sani, 2018)
Acetone	Antiproliferative effect against Non-Hodgkin's lymphoma cells by decreasing their mitochondrial membrane potential, increasing	(Lu et al., 2018)

	reactive oxygen species and calcium ions, and upregulating the IRE-la and CHOP proteins.	
Chloroform	Antiproliferative effects against erythroleukemia (K-562), cervical cancer (HeLa), gastric cancer (SNU-1), lung cancer (NCI-H23), colon adenocarcinoma (LS-174T), hepatocellular carcinoma (HepG2), and Burkitt's lymphoma (Raji).	(Yong et al., 2013)
Chloroform and hexane	Antiproliferative effects on HepG2, nasopharyngeal cancer (CNE1) and non-small cell lung cancer (A549). Hexane extract induced sub-G1 phase arrest and oxidative stress-induced apoptosis. Upregulation of caspases 8 and 9 was observed at high concentrations (> 100 µg/mL).	(Ng et al., 2017)
Ethanol	Neuroprotective effects on endothelial cells and astrocytes under hypoxic conditions by downregulating histone deacetylases (HDACI/6).	(Tsai et al., 2016)
Ethanol	Antioxidant activity (protective effect) of plasmid DNA from riboflavin photoreaction.	(Yuann et al., 2012)
Ethanol and ethyl acetate extracts	Antiproliferative effects on breast cancer cells (MCF-7).	(Intan et al., 2015)
Hexane, dichloromethane and methanol	Antiviral activities against HSV-1 and HSV-2.	(Kunsorn et al., 2013)
Methanol	Anticancer effect by reducing nitric oxide and malondialdehyde levels in the blood of mice treated with 200 mg/kg and 1,000 mg/kg of leaf extract. Mitotic cells, tumour weight, and volume were decreased. No adverse or inflammatory reactions related to splenocyte activities were observed in all treated mice.	(Haron et al., 2019)
Methanol	Cytotoxic effects were found against HepG2, A549, HT-29, MDA-MB-231, MCF-7, and CRL-1739 cancer cell lines. Inhibitory activity against Cytochrome P450 (CYP3A4 & CYP2E1) in human liver microsomes.	(Quah et al., 2017)
Methanol	Induced apoptosis in human melanoma cells (D24).	(Fong et al., 2016)
Methanol	Central and peripheral antinociceptive activity by activating the opioid receptor and nitric oxide-mediated pathway.	(Abdul Rahim et al., 2016)
Methanol	Inhibited the growth of MDA-MB-231 triple-negative breast cancer cell line.	(Khiru Nasir & Mohd Bohari, 2015)
Methanol	Increased AChE activity in the liver, kidney and heart but not in the brain of male mice.	(Lau et al., 2014)
Methanol	Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus.*	(Yang et al., 2013)

Methanol and water	Termination of hyperlipidemia-associated oxidative stress in rats by increasing the activity of antioxidant enzymes as well as the expression of antioxidant genes in serum and liver.	(Sarega et al., 2016)
Petroleum ether and ethyl acetate	Only petroleum ether leaf extract inhibited the growth of HeLa cells.	(Arullappan et al., 2014)
Water	Sonicated water extract had the highest nitric oxide inhibitory effect in lipopolysaccharide-interferon-gamma-induced RAW 264.7 macrophages.	(Khoo et al., 2019)
Water	Anti-angiogenic activities in endothelial cells.	(Ng et al., 2018)

2.2 Carbohydrates

The four major classes of organic compounds are carbohydrates, lipids, proteins, and nucleic acids. These compounds are known as polymers or macromolecules and play a vital role in the proper functioning of living organisms (Albuquerque et al., 2020). These four compounds are formed primarily by different carbon, hydrogen, and oxygen ratios. Among these, carbohydrates are the most abundant in nature. They are majorly produced by plants and algae all year round (Xu & Queneau, 2014; Albuquerque et al., 2020). Carbohydrates are formed by the conversion of water and carbon dioxide into energy during the photosynthesis of green plants. Generally, they serve as an energy source and a structural component of organisms. Aside from being an energy source, carbohydrates are also known to contain genetic information, making up a part of nucleic acid (Davidson, 2018).

Carbohydrates simply mean ‘carbon’ and ‘water’, which contributes to the empirical formula of the simplest carbohydrate; $[C(H_2O)]_n$, where n = 3 to 7 (Subrata Pal, 2020; Seeberger, 2022). Carbohydrates fall into three major structural classes: monosaccharides, oligosaccharides, and polysaccharides. These three classes are according to the number of structural units that they contain (Ouellette & Rawn, 2015).

The simplest carbohydrate that cannot be hydrolysed into smaller molecules is known as monosaccharides. These carbohydrate molecules have about three to six carbon atoms. The compounds may be either aldehydes, ketones, acetals or ketals, which yield a monosaccharide and alcohol when hydrolysed (Ouellette & Rawn, 2015). Oligosaccharides, however, are typically a few monosaccharides combined: 2 - 10. The glycosidic linkages (acetal or ketal) in oligosaccharides are broken with hydrolysis, which releases the monosaccharide units. Oligosaccharides are also known as disaccharides, trisaccharides, tetrasaccharides and so on, differing with the number of linked monosaccharide units (Ouellette & Rawn, 2015).

On the other hand, polysaccharides are usually composed of thousands of monosaccharide units linked covalently. Each polysaccharide contains a central unit that serves as the acetal/ketal centre to form a glycosidic bond to the neighbouring monosaccharide (Ouellette & Rawn, 2015). Generally, polysaccharides are divided into two subunits, homopolysaccharides or heteropolysaccharides. Homopolysaccharides are made up of only one type of monosaccharide. Heteropolysaccharides are characterised by multiple types of monosaccharides (Ouellette & Rawn, 2015). Both polysaccharides are formed by the monosaccharide units either linking in a linear fashion or branching out to form complex sugars (Xie et al., 2016).

2.2.1 Crude polysaccharides

Despite the widely known properties of simple carbohydrates, the complex structure of polysaccharides remains an area of interest. In the process of isolation/ extraction, polysaccharides rarely exist on their own; rather, it exists as a conjugate with other components which act as a whole (Junqiao Wang et al., 2016). Hence, crude

polysaccharides may contain small amounts of protein, phenolic compounds, and uronic acid conjugates (Ross et al., 2015).

Uronic acids are monosaccharides where the terminal hydroxymethyl group has been oxidised to a carboxylic acid. They are also essential components of many life forms, involved in the formation of various polysaccharides and glycoconjugates (Biermann, 2021; Boleij et al., 2020). Phenolic compounds are a major secondary metabolite found in plants and are separated into two; phenolic acids and polyphenols. Mono- and polysaccharides are sometimes found combined with one or more phenolic compounds or even appear as a derivative (ester or methyl esters) (Minatel et al., 2017).

Generally, the bioactivities of crude polysaccharides outweigh the bioactivities of purified polysaccharides. There have been claims that the antioxidant activity of crude polysaccharides was reduced dramatically upon fractionation and purification of the plant polysaccharide. These studies suggested the possibility that the other compounds present in the crude polysaccharide extract (such as pigments, proteins and polyphenols) were contributing to the higher antioxidant activity (Fan et al., 2014; Wu et al., 2014).

Crude polysaccharides also showed pronounced antioxidant activity in DPPH radical scavenging assays, which could be due to the polyphenolic-associated polysaccharide fraction that is formed between the polysaccharides and the high molecular weight phenolics. A study revealed that the phenolic and protein content of three different mushroom species (*Lentinula edodes, Grifola frondosa,* and *Trametes versicolor*) were significantly correlated with elevated antioxidant activities (Siu et al., 2014). Nevertheless, phenolic and protein-free purified polysaccharides barely showed antioxidant activities. Hence, crude polysaccharides with some amount of phenolic

compounds conjugated might be the reason for the increased antioxidant potential compared to purified polysaccharides.

Additionally, scientific investigations related to polysaccharide technology are needed to develop new approaches for the exploitation of its potential in the development of biocomposite, nano-conjugate, and pharmaceutical delivery systems. Even though different formulations may have different properties, polysaccharides are still superior to other polymers when it comes to terms of ease of use, non-toxicity, biocompatibility, and biodegradability (Gopinath et al., 2018).

2.2.1(a) Extraction of water-soluble crude polysaccharides from plants

Water extraction of polysaccharides is the most common, easily accessible, and safe method of extraction used in the food industry (Jing et al., 2016). Basically, the efficiency of polysaccharide extraction and yield is influenced by several factors, including the extraction time, temperature and water-to-extract ratio (Shang et al., 2017). Most plant polysaccharides are composed of structural constituents that can be extracted from the cell walls. The various methods used for extracting these constituents vary depending on the structure of the cell (Chen et al., 2021). Some commonly used water extraction methods include hot water extraction, acidic extraction, and dilute alkaline extraction method.

Recently, enzymatic and physical extraction methods, including freeze-thaw, microwave and ultrasonic extraction methods, have also been applied with much success in the extraction of plant polysaccharides (X. Ma et al., 2017). Then the isolation of crude polysaccharides is done by alcohol precipitation. Since polysaccharides are not soluble in alcohol, further washing steps done with pure alcohol would remove residues from the crude polysaccharide. The percentage yield

and the bioactivity of all plant polysaccharides vary based on the extraction and isolation procedure used (Chen et al., 2021).

Among the extraction methods, the ultrasound-assisted extraction method is commonly used for the extraction of various substances. Due to its numerous physical effects, ultrasound waves could effectively and quickly diffuse into the plant cell and release the active components into the solvent, making it favourable in the extraction of polysaccharides. Generally, ultrasound-assisted extraction improves the extraction rate and extraction time (X. Ma et al., 2017). This was attributed to the fact that ultrasonication increased the protein content in crude polysaccharides (Mtetwa et al., 2020).

Alkaline and acidic extraction methods yield alkaline-soluble and acid-soluble polysaccharides, respectively. A number of studies suggested that acidic extraction methods allowed higher yields of low molecular weight polysaccharides by hydrolysing the glycosidic bonds (Gao et al., 2017; Yao et al., 2017; Sun et al., 2018). Nevertheless, there are several studies that suggest the alkaline extraction method is efficient in obtaining polysaccharides rich in bioactivities (Zhang et al., 2013; Khodaei et al., 2016; Ma & Mu, 2016). A study conducted using polysaccharides extracted from *Lonicera japonica* (LJP) reported that the extraction yield of the polysaccharides from an alkaline solution was higher than that of acidic or water extraction methods (Sun et al., 2018). This study suggested that all three extractions (LJP- Acid, LJP-Alkali and LJP-Water) were rich in uronic acid content and fucose but had different molecular weights, structures, colours and were made up of different monosaccharides. Besides, LJP-Alkali exhibited the most advantageous properties when subjected to ion chelating studies on egg yolk homogenates, suggesting the inhibition of lipid peroxidation (Zhu et al., 2016). Based on several studies, crude polysaccharides

extracted via alkaline and acidic methods seem to have more potent biological activities and produce a higher yield of polysaccharides (Sun et al., 2018).

One study conducted to explore the impact of the extraction pH on the extracted polysaccharide yield from peanut sediment of an aqueous extraction process reported that the yield was significantly higher at acid (pH 4) and alkali (pH 12). As the pH decreased from 2 - 6, the yield increased. At acidic pH (pH 4), a breakage of the biopolymer glycosidic bonds by hydrogen ions from HCl was the main reason for the dissolving of the polysaccharides. However, at pH 6, the temperature was predominantly responsible for breaking the biopolymer structures. Moreover, an increase in the extraction pH from 8 to 12 enhanced the dissolution of proteins and thereby raised the yield (Ye et al., 2019).

2.2.1(b) Solubility

Monosaccharides are polar molecules with several -OH functional groups that are responsible for their hydrophilic nature. Besides, monosaccharides have a lower solubility in organic solvents compared to water (Istasse & Richel, 2020). Polysaccharides, however, have a diverse range of solubility; some are water-insoluble (cellulose), some are insoluble in cold water (starch); and some are insoluble in hot water (pullulan and gum Arabic) (Guo et al., 2017). Nevertheless, due to the multi-OH groups, polysaccharides have a strong affinity for water molecules. This can cause polysaccharides to interact with each other through hydrogen bonding. Polysaccharide solubility depends solely on the interaction between molecule-molecule and molecule-solvent. In water-soluble polysaccharides, the favourable interaction is between water and polysaccharide molecules, where the solvent forms a solvating envelope enclosing the polymer chain. This causes the polysaccharide molecules to be apart from each other (Guo et al., 2017).

For poorly water-soluble polysaccharides, the intermolecular interactions between the polysaccharide polymer segments can lead to aggregation and eventually to gelation. Under certain conditions, the interaction between the polysaccharide polymers and the water molecules can compensate for the polymer-water interaction; this is known as the theta condition. In theta condition, the chain conformation is defined by the bond angles and the short-range interactions that occur between the polymer coil dimension and the bond (Guo et al., 2017). The solubility of polysaccharides depends on their molecular structures. Higher solubility is linked to structural features that inhibit the formation of intramolecular associations, which includes charged groups (carboxylate, phosphate and sulfate groups) and branching structures. In contrast, lower solubility is linked to structural features that elevate the intramolecular associations, including large molecular weight and linear chain (Guo et al., 2017).

Therefore, polysaccharide structural features play a crucial role in determining the solubility of the sugar. In an ethanol-water solution, the soluble properties of the polysaccharides decreased as the ethanol content increased. The results also showed that the soluble properties of the oligosaccharides were minimal in pure ethanol (Guo et al., 2017). This explains the precipitation and washing steps of carbohydrates using pure ethanol during the extraction procedure.

2.2.1(c) Antioxidant activities

The presence of protein and pigments in alkaline-soluble and water-soluble crude polysaccharides from *Inonotus obliquus* could influence the radical scavenging effects (Mohanta et al., 2022). It has also been hypothesised that the radical scavenging ability of polysaccharide molecules is linked to the number of hydroxyl groups in their polysaccharides (Zhao et al., 2013). Numerous studies reported that crude

polysaccharide extracts exhibited potent antioxidant activities as compared after further fractionation, which yielded purified polysaccharides. Purified polysaccharides yield low to moderate activity, which suggests that the presence of a small number of other plant compounds, including proteins and polyphenols, contributes to the increased antioxidant activity (Cheng & Huang, 2018; Hamed et al., 2020; Huo et al., 2022).

In 2013, a study on the crude polysaccharides of tea extracts was tested to identify the role of the polyphenols present in order to evaluate the antioxidant capabilities. This study concluded that the crude tea polysaccharides exhibited potent antioxidant activities as compared to the further purified polysaccharide fractions, which hardly exhibited any antioxidant activity. This study suggested that in the presence of polyphenols, the DPPH radical scavenging ability was enhanced, indicating that polyphenols triggered a synergistic elevation in the antioxidant activity (Wang et al., 2013). Another study conducted to compare the antioxidant activity of crude, and purified fractions of polysaccharides from the plant *Dendrobium officinale* reported that the DPPH scavenging activity was significantly higher in crude polysaccharides. They suggested the reason for the higher antioxidant content could possibly be due to the presence of other compounds, including polyphenols, proteins and microelements (Zhao et al., 2013). A similar study was conducted, with similar results, indicating the increase in antioxidant activity in crude polysaccharides from *Fritillaria pallidiflora* could possibly be due to the protein present in them (Rozi et al., 2019).

Furthermore, acidic extracted crude polysaccharides are considered efficient antioxidants due to the presence of uronic acids, which are believed to be key components contributing to the antioxidant activity in polysaccharides (Al-Sheraji et

al., 2012; Hui et al., 2013). Acidic polysaccharides has a higher concentration of hydrogen ions making aldehydes or ketones oxidise easily (Jiang et al., 2021). One study elucidated that the antioxidant activity, including DPPH radical scavenging and FRAP on glucuronic acid, galacturonic acid, and polygalacturonic acid, exhibited potent antioxidant activity, with the highest activity reported in polygalacturonic acid. The researchers concluded that the nature of the polymerisation might be the reason for the difference in activity (Rao & Muralikrishna, 2006).

2.3 Metabolomics

The omics approach and hierarchy are shown in Figure 2.2. Metabolomics is a scientific study of the products and substrates of metabolism. The metabolism of a living organism is affected by both its environment and genetic factors. Therefore, metabolomics is a quantitative technique that allows the identification and quantitation of the various metabolites that play a role in the metabolism of living organisms (Alseekh et al., 2021; Clish, 2015).

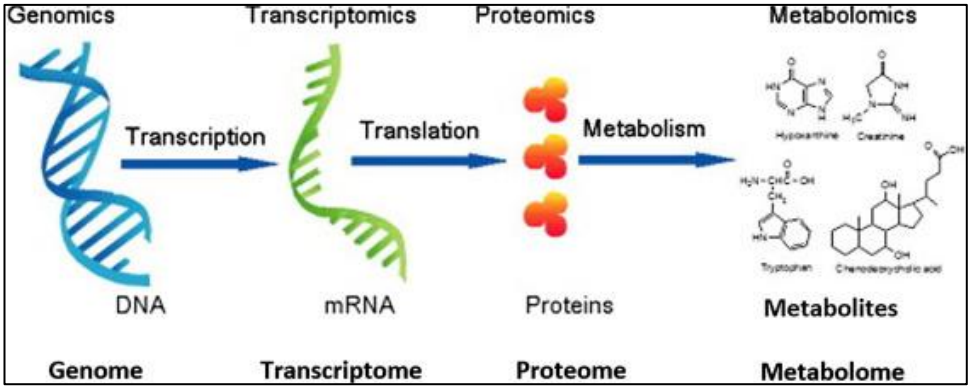


Figure 2.2 Omics approach and hierarchy. Extracted from Frueh & Burczynski (2020).

Metabolomics is an upcoming field used for various applications; including for comparing mutants (Clark et al., 2019), assessing responses to environmental stress (L. J. Zhang et al., 2021), studying global effects of genetic manipulation (Bragagnolo

et al., 2021), toxicology evaluations (Olesti et al., 2021), nutritional epidemiology (Brennan et al., 2021; Levatte et al., 2022), cancer pharmacology (Liang et al., 2021), drug discovery (Tounta et al., 2021; Alarcon-Barrera et al., 2022) and natural product discovery (Caesar et al., 2021; Tsugawa et al., 2021). There are three main approaches used for metabolomics studies: metabolic profiling which is divided into targeted analysis and un-targeted analysis, as well as metabolic fingerprinting (Onuh & Qiu, 2021). The advantages and disadvantages of these major approaches are shown in Table 2.2. Targeted approaches require prior knowledge of the molecule(s) of interest to design tailored analytical methods. Nevertheless, the establishment of extensive targeted methods has been facilitated by the increasing availability of metabolite libraries (e.g., IROA technologies) (Han et al., 2021). Untargeted approaches aim to detect as many metabolites as possible in a sample with subsequent annotation and identification, usually involving database searches (e.g., METLIN or MS-DIAL) (Tsugawa et al., 2015) as well as comparison with authentic synthetic materials (Salek et al., 2013).

Table 2.2 Advantages and disadvantages of the major approaches in metabolomics. Adapted from Fraga-Corral et al. (2022).

Approach	**Advantage**	**Disadvantage**
Metabolic profiling (targeted)	• Quantitative • Low limit of detection • High throughput	• Limited number of compounds can be targeted • Does not detect compounds that were not targeted • Targeted compounds must be available and purified for calibration

Metabolic profiling (un-targeted)	• Global (not targeted)	• Semi-quantitative • Majority of peaks are not identifiable • Difficult informatics • Medium throughput
Metabolic fingerprinting	• Global (not targeted) • Directly applicable to pattern recognition • Highest throughput	• No compound identification

Plant metabolomics is a branch of metabolomics designed to study the overall changes in a large number of metabolites in plant samples and then conduct deep data mining and bioinformatic analysis (Xiao et al., 2022). Plant metabolites are components of defence systems developed by plants in response to pathogen attacks and other environmental stresses. They are an important source of many natural pharmacological activity products which are often designated as specific biological activities associated with their biochemical structures (Lu & Cui, 2019).

Metabolite profiling is the commonest technique used for assessing the level of metabolites in samples. The identification of plant metabolites is associated with a series of events, including the experimental design, sample collection, sample processing, sample preparation, detection, analysis and data processing (Salem et al., 2020). Analytical techniques and data processing techniques are the two major parts of metabolomics. Table 2.3 illustrates the analytical techniques together with their advantages and disadvantages in metabolite profiling. The data processing techniques are reviewed in Section 2.8.2 (b).