

**SYNTHESIS, CHARACTERISATION AND CYTOTOXIC
EVALUATION OF SILVER NANOPARTICLES FROM *Euphorbia
mili* LEAVES**

NUR RASYIDAH BINTI RAMLI

**MASTER OF SCIENCE
UNIVERSITI MALAYSIA TERENGGANU**

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MASTER OF SCIENCE

2026

**SYNTHESIS, CHARACTERISATION AND CYTOTOXIC EVALUATION
OF SILVER NANOPARTICLES FROM *Euphorbia milii* LEAVES**

NUR RASYIDAH BINTI RAMLI

**Thesis Submitted in Fulfilment of the Requirement for the
Master of Science
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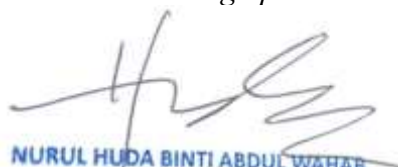
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Dedicated this thesis to:

My all: Abah & Ibu.

My husband: Irfan

My anchor throughout the storm & turbulence:

Atikah, Firzana, Zaleha, Amirah, Syifa, Syazwani, Izzati & Ammar

I owe you guys, big time

Thanks a lot

May Allah bless

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in
fulfilment of the requirements for the degree of Master of Science

**SYNTHESIS, CHARACTERISATION AND CYTOTOXIC EVALUATION
OF SILVER NANOPARTICLES FROM *Euphorbia milii* LEAVES**

NUR RASYIDAH BINTI RAMLI

MAY 2025

Main Supervisor : Nurul Huda Abdul Wahab
Co-Supervisor : Suzana Misbah, Ph.D
Faculty : Faculty of Science and Marine Environment

Silver nanoparticles (AgNPs) can be synthesised through chemical, physical, or green approaches, with plant-mediated synthesis offering a safer and more sustainable alternative. This study employed a green synthesis method using *Euphorbia milii* (*E. milii*) leaf extract as a natural reducing and stabilising agent. *Euphorbia milii*, commonly known as the crown-of-thorns, was selected due to its high flavonoid content, which contributes to nanoparticle reduction efficiency and stability. Leaf extracts obtained using solvents of different polarity were chemically profiled through Total Flavonoid Content (TFC), Nuclear Magnetic Resonance (NMR), and High-Performance Liquid Chromatography (HPLC) analyses to identify their major flavonoid constituents. These extracts, alongside pure flavonoids (rutin, vitexin, and quercetin), were reacted with 1 mM AgNO₃ to synthesise AgNPs under dark conditions at room temperature. AgNPs formation and stability were characterised using UV–Visible Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscopy (SEM). UV–Vis spectra showed surface plasmon resonance (SPR) peaks between 415–485.5 nm, indicating successful nanoparticle formation. FTIR analysis revealed functional groups such as O–H (3333 cm⁻¹), C=C (1636 cm⁻¹), and aromatic C–H (664 cm⁻¹), confirming the participation of flavonoids in nanoparticle reduction and capping. SEM micrographs demonstrated particle sizes of 67–843 nm for crude extracts and 117–324 nm for pure flavonoids, with varying degrees of agglomeration. Cytotoxicity was evaluated using the MTT assay on Vero

cells. AgNPs–rutin showed improved biocompatibility compared to pure rutin, with significantly higher cell viability at $1000\ \mu\text{g mL}^{-1}$ ($73.2 \pm 2.8\%$ vs. $61.5 \pm 3.1\%$, $p < 0.05$). Both formulations maintained $>85\%$ viability at moderate concentrations ($31.25\text{--}125\ \mu\text{g mL}^{-1}$). Overall, the findings demonstrate that *E. milii* extract and rutin are effective biogenic agents for AgNP synthesis. The resulting AgNPs–rutin exhibit enhanced physicochemical stability and reduced cytotoxicity, highlighting their potential application as a safer nanocarrier system in biomedical fields.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

**SINTESIS, PENCIRIAN DAN PENILAIAN KETOKSIKAN
NANOPARTIKEL PERAK DARI DAUN *Euphorbia milii***

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Nanopartikel perak (AgNPs) boleh disintesis melalui kaedah kimia, fizikal atau biosintesis, dengan kaedah hijau berasaskan tumbuhan, menjadi alternatif yang lebih selamat dan mesra alam. Kajian ini menggunakan kaedah biosintesis hijau dengan ekstrak daun *Euphorbia milii* (*E. milii*) sebagai agen penurun dan penstabil semula jadi. *Euphorbia milii*, atau Mahkota Duri, dipilih kerana kandungan flavonoidnya yang tinggi yang menyumbang kepada kecekapan penurunan serta kestabilan nanopartikel. Ekstrak daun yang diperoleh menggunakan pelarut dengan polariti berbeza diprofil secara kimia melalui analisis Jumlah Kandungan Flavonoid (TFC), Resonans Magnet Nuklear (NMR) dan Kromatografi Cecair Berprestasi Tinggi (HPLC) bagi mengenal pasti sebatian flavonoid utama. Ekstrak ini bersama flavonoid tulen (rutin, vitexin dan quercetin), kemudiannya ditindakbalaskan dengan 1 mM AgNO₃ untuk mensintesis AgNPs di bawah keadaan gelap pada suhu bilik. Pembentukan dan pencirian AgNPs disahkan melalui Spektroskopi Ultralembayung-Nampak (UV-Vis), Spektroskopi Inframerah Transformasi Fourier (FTIR), dan Mikroskop Pengimbasan Elektron (SEM). Analisis UV-Vis menunjukkan kehadiran puncak permukaan plasmon resonans (SPR) antara 415–485.5 nm, mengesahkan pembentukan AgNPs. Spektrum FTIR menunjukkan kehadiran kumpulan berfungsi seperti O–H (3333 cm⁻¹), C=C (1636 cm⁻¹) dan C–H aromatik (664 cm⁻¹), sekali gus membuktikan penglibatan flavonoid dalam proses penurunan dan penstabilan. Mikrograf SEM menunjukkan saiz partikel antara 67–843 nm bagi ekstrak mentah dan 117–324 nm bagi flavonoid tulen, dengan tahap aglomerasi yang berbeza. Kesitotoksikan dinilai menggunakan ujian

MTT terhadap sel Vero. AgNPs–rutin menunjukkan biokeserasian yang lebih baik berbanding rutin tulen, dengan viabiliti sel lebih tinggi pada $1000 \mu\text{g mL}^{-1}$ ($73.2 \pm 2.8\%$ berbanding $61.5 \pm 3.1\%$, $p < 0.05$). Kedua-dua formulasi mengekalkan viabiliti melebihi 85% pada kepekatan sederhana ($31.25\text{--}125 \mu\text{g mL}^{-1}$). Secara keseluruhan, dapatan ini menunjukkan bahawa ekstrak *E. milii* dan rutin merupakan agen biogenik yang berkesan untuk sintesis AgNPs. AgNPs–rutin yang dihasilkan mempunyai kestabilan fizikokimia yang lebih baik dan kesitotoksikan yang lebih rendah, sekaligus menunjukkan potensinya sebagai sistem pengangkut nano yang lebih selamat untuk aplikasi bioperubatan.

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I would like to express my heartfelt appreciation to my beloved parents for their unwavering support and encouragement throughout my academic journey. Their love and guidance have been instrumental in shaping the person I am today.

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APPROVALS

I certify that an Examination Committee has met on 13rd May 2025 to conduct the final examination of Nur Rasyidah Binti Ramli, on her Master of Sciences thesis entitled **“SYNTHESIS, CHARACTERISATION AND CYTOTOXIC EVALUATION OF SILVER NANOPARTICLES FROM *Euphorbia milii* LEAVES”** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.



NUR RASYIDAH BINTI RAMLI

Date: 14/8/2026

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
<i>E. milii</i>	<i>Euphorbia milii</i>
Ag	Silver
Au	Gold
HPLC	High-Performance Liquid Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
NMR	Nuclear Magnetic Resonance
AgNO ₃	Silver nitrate
XRD	X-ray diffraction
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
UV-Vis	UV-Visible Spectroscopy
MCF-7	breast cancer
A549	lung cancer
HeLa	cervical cancer
ROS	reactive oxygen species
AuNPs	Gold nanoparticles
ZnONPs	Zinc oxide nanoparticle
PEG	polyethylene glycol
DMSO	Dimethylsulfoxide
PBS	phosphate buffer saline
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
ITO	Indium Tin Oxide

TFC	Total Flavonoid Content
ACN	Acetonitrile
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PenStrep	Penicillin-Streptomycin
NEAA	Non-Essential Amino Acids
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
SPR	surface plasmon resonance
SH	AgNPs mediated with extract hexane
SC	AgNPs mediated with extract chloroform
SE	AgNPs mediated with extract ethylacetate
SA	AgNPs mediated with extract acetone
SM	AgNPs mediated with extract methanol
SD	AgNPs mediated with extract distilled water
SR	AgNPs mediated with Rutin
SV	AgNPs mediated with Vitexin
SQ	AgNPs mediated with Quercetin

LIST OF SYMBOLS

%	Percentage
V	Volume
t	Time
nm	Nanometre
ml	Millilitre
min	Minute
h	hour
g	Gram
°C	Degree Celsius
ppm	Parts per million
mg	Milligram
QE/g	Quercetin equivalent per gram
cm ⁻¹	Reciprocal centimeter
a.u	Unitless quantity
µg/mL	Microgram per mililitre
µL	Microlitre

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Nanoparticles are extremely small materials, generally measuring between 1 and 100 nanometres (nm), that exhibit distinctive physicochemical characteristics compared to their bulk forms. These differences arise mainly from their large surface-area-to-volume ratio and quantum confinement effects, which influence their chemical reactivity and functional properties (Bhattacharyya et al., 2010; Jeevanandam et al., 2018). Among various nanomaterials, silver nanoparticles (AgNPs) have received considerable attention in nanotechnology because of their remarkable antimicrobial, antioxidant, and anticancer activities (Abass Sofi et al., 2021). Their nanoscale dimensions and extensive surface area enhance their interaction with biological systems, making them highly effective in diverse biomedical applications (Abbas et al., 2024). Nevertheless, conventional chemical synthesis routes for AgNPs often involve toxic reagents and high energy consumption, which raise safety and environmental concerns (Vishwananth & Negi, 2021).

The green synthesis approach provides an environmentally friendly method for producing silver nanoparticles (AgNPs) by utilizing plant-based biomolecules as natural reducing and stabilizing agents (Ying et al., 2022). Plants contain a diverse range of secondary metabolites, including flavonoids, phenolics, and alkaloids, which exhibit strong antioxidant properties and play a key role in converting silver ions into

nanoparticles (Fahim et al., 2024). Among these compounds, flavonoids stand out due to their ability to chelate metal ions and their structural variability, both of which significantly impact the size, shape, and stability of the resulting nanoparticles (Abdi Syahputera et al., 2024).

E. milii, also known as the Crown of Thorns, has long been recognized for its medicinal benefits, particularly its antimicrobial and anti-inflammatory properties (Mutalib et al., 2020). Its leaves contain abundant flavonoids, which makes it a strong contender for eco-friendly nanoparticle synthesis. However, while the plant's bioactive potential is well-documented, research on its role in silver nanoparticle production remains limited.

This study seeks to bridge this gap by identifying the specific flavonoid compounds in the methanol extract of *E. milii* leaves that are most compatible as reducing agents for synthesizing stable and effective silver nanoparticles. Through chemical profiling, green synthesis, and cytotoxicity assays, the research aims to contribute to the development of environmentally friendly nanomaterials with enhanced therapeutic applications, aligning with the principles of green chemistry and sustainable development. Figure 1.1 shows the leaves of *E. milii*.



Figure 1.1 Leaves of *Euphorbia milii*

1.2 Problem Statement

The synthesis of silver nanoparticles (AgNPs) is widely studied due to their diverse applications in medicine, particularly for their antimicrobial and anticancer properties (Fahim et al. 2024). However, traditional chemical synthesis methods often involve toxic reagents and produce nanoparticles with limited stability and biocompatibility (Dhaka et al. 2023). The green synthesis approach, utilizing plant-derived biomolecules, addresses these challenges by providing an eco-friendly and sustainable alternative.

Despite these advancements, identifying specific plant-derived compounds that optimize the stability, size, and biological activity of AgNPs remains a significant challenge. *E. milii*, known for its rich flavonoid content, holds potential for producing highly stable and effective AgNPs (Bawazeer, 2024). However, the compatibility of individual flavonoid compounds within the plant extract as reducing agents for AgNPs synthesis has not been fully explored. This gap necessitates a systematic study to identify the most effective flavonoids for producing stable AgNPs with enhanced cytotoxicity properties for medicinal health applications. Figure 1.2 showed the Illustration about this study.

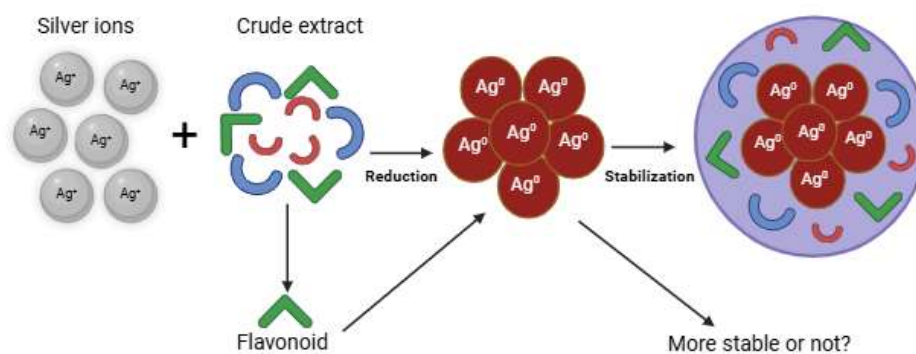


Figure 1.2 Illustration of synthesis AgNPs using both crude extract and pure compound

1.3 Scope of Study

This study focuses on the green synthesis of silver nanoparticles (AgNPs) using flavonoid compounds extracted from the methanol extract of *E. milii* leaves. The scope includes the chemical profiling of flavonoid compounds in the plant extract to identify potential reducing agents for nanoparticle synthesis. The synthesis process will involve optimizing parameters to ensure the production of stable and effective nanoparticles. The synthesized AgNPs will then be characterized for their physicochemical properties, such as size, shape, stability, and surface chemistry.

In addition, the study involves the assessment of the cytotoxic activity of the synthesized AgNPs to explore their potential for medicinal applications. The scope of the research is confined to *E. milii* leaf methanol extracts, excluding other plant parts or solvent systems. This study is limited to in vitro cytotoxicity assessment using a single cell line (Vero cells) and does not include in vivo experiments or clinical evaluations.

1.4 Research Objective

The objectives of the research are listed as below:

- i) To chemically profile the flavonoid compounds, present in the methanol extract of *Euphorbia milii* leaves.
- ii) To synthesize silver nanoparticles using the identified flavonoids as reducing agents through a green synthesis approach.
- iii) To evaluate the stability and physicochemical properties of the synthesized silver nanoparticles.
- iv) To assess the cytotoxic activity of the synthesized silver nanoparticles using statistical analysis (one-way ANOVA and independent t-tests)

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

2.1.1 Overview of Green Nanotechnology in Biomedical Applications

Green nanotechnology has gained considerable attention in biomedical research due to its sustainable approach and reduced reliance on hazardous chemicals (Kazemi et al. 2023). This field emphasizes environmentally friendly methods for nanoparticle synthesis, utilizing natural resources such as plant extracts and microorganisms (Ying et al. 2022). By eliminating the need for toxic chemical reagents, green synthesis offers a safer and more eco-conscious alternative for the production of nanoparticles with potential biomedical applications (Noah & Ndangili 2022).

Figure 2.1 illustrates the various green synthesis techniques employed to produce nanoparticles, including physical, chemical, and biological approaches (Ying et al. 2022). These eco-friendly nanoparticles can be synthesized using resources like plant extracts, microorganisms, enzymes, agricultural waste, and methods assisted by ultrasound and microwave technologies (Rathod et al. 2024). Resulting nanoparticles, such as liposomes, niosomes, nanoemulsions, and nanogels, have broad applications across diverse sectors, including agriculture, biomedicine, environmental science, food and feed industries, and energy production.

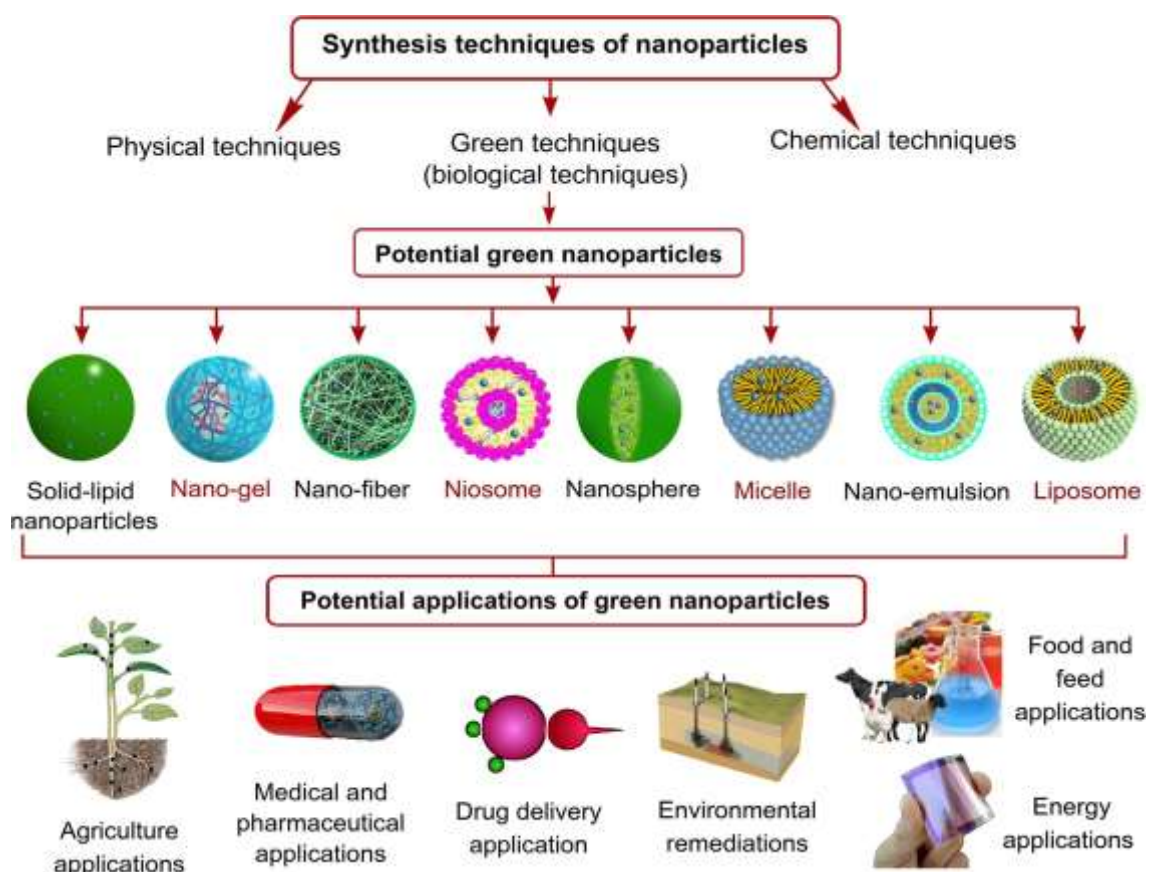


Figure 2.1 Nanoparticle synthesis and applications (Osman et.al, 2024)

Recent research has highlighted the effectiveness of green-synthesized nanoparticles in various biomedical fields. Osman et al. (2024) particularly emphasized their potential in sustainable development, including environmental cleanup, agriculture, and medicine. In the medical field, green-synthesized silver (Ag) and gold (Au) nanoparticles have been extensively investigated for their antimicrobial and anticancer properties. Sharma et al. (2023) explored the eco-friendly production of silver (Ag) and gold (Au) nanoparticles, emphasizing their potential applications in medicine, drug delivery, biosensing, and diagnostics.

Furthermore, the integration of green nanotechnology in drug delivery systems has shown promising results. Noah and Ndangili (2022) discussed the green synthesis of nanoparticles derived from sustainable sources for applications in biosensors and targeted drug delivery. Their findings underscore the biocompatibility and efficacy of these nanomaterials, highlighting their potential in enhancing treatment precision while minimizing toxicity.

Overall, green nanotechnology presents a sustainable and effective strategy for the development of nanomaterials in biomedical applications. By aligning with environmental conservation efforts and advancing human health, green synthesis offers a promising pathway toward safer and more efficient nanomedicine (Kazemi et al. 2023).

2.1.2 Importance of Plant-Mediated Synthesis of Nanoparticles

Bhardwaj et al. (2020) highlighted that using plants to synthesize nanoparticles has emerged as a safer and more sustainable alternative to traditional chemical and physical methods. Conventional nanoparticle production often relies on harmful reducing agents like sodium borohydride and hydrazine, which pose serious risks to both the environment and human health (Fahim et al., 2024). In contrast, green synthesis harnesses naturally occurring biomolecules from plant extracts such as flavonoids, tannins, and polyphenols to create nanoparticles without toxic byproducts (Iravani et al., 2015). This eco-friendly approach not only minimizes waste and reduces energy consumption but also aligns with the principles of green chemistry.

One of the primary advantages of plant-mediated synthesis is its cost-effectiveness and scalability (Ahmad et al. 2021). Unlike chemical methods that require specialized equipment and stringent reaction conditions, green synthesis can be conducted under mild conditions, such as ambient temperature and neutral pH (Shayo et al. 2024). Additionally, plant extracts simplify the process by serving as both stabilizing and reducing agents, eliminating the need for multiple chemical additives (Ahmed et al., 2016). Their widespread availability and abundance make large-scale production more feasible, making this approach particularly attractive for industrial applications.

Moreover, plant-derived nanoparticles offer enhanced biological activity and biocompatibility, making them well-suited for biomedical applications (Karnwal et al., 2024). Beyond simply aiding in nanoparticle formation, the natural bioactive compounds they contain such as flavonoids and polyphenols also provide additional therapeutic benefits, including antibacterial and anticancer properties (Mittal et al., 2013). These natural compounds contribute to the stability of nanoparticles, preventing

aggregation and ensuring prolonged activity (Phan & Haes 2019). Given these advantages, plant-mediated synthesis has gained significant attention in the fields of nanomedicine, drug delivery, and biosensing (Sharma et al. 2019).

2.1.3 Relevance of *Euphorbia milii* in nanotechnology

E. milii, also known as the Crown of Thorns, has gained significant attention in nanotechnology due to its strong reducing properties and rich phytochemical composition (Bawazeer et al., 2024). This plant contains a variety of bioactive compounds, including flavonoids, alkaloids, tannins, and terpenoids, which play a key role in the eco-friendly synthesis of nanoparticles (Hasan et al., 2020). These phytochemicals serve as natural reducing and stabilizing agents, allowing for the eco-friendly synthesis of AgNPs without the need for hazardous chemicals (Farooqi et al. 2024). The use of *E. milii* in nanoparticle synthesis aligns with sustainable and green chemistry principles, making it a promising alternative to conventional synthetic methods.

Several studies have demonstrated that extracts from *E. milii* can produce AgNPs with desirable physicochemical properties, such as controlled size, stability, and enhanced bioactivity (Bawazeer et al. 2024). The presence of flavonoids and polyphenols in the plant extract not only facilitates nanoparticle formation but also enhances their biological functionalities, including antimicrobial, antioxidant, and anticancer properties (Rajakumar et al. 2017). Due to its biocompatibility, *E. milii*-mediated AgNPs have been explored for applications in drug delivery, biosensing, and wound healing.

Beyond biomedical applications, *E. milii*-based nanoparticles have shown promise in environmental remediation and catalysis. The high stability and reactivity of these nanoparticles make them suitable for applications such as water purification, heavy metal ion removal, and degradation of toxic pollutants (Subramani et al. 2018). With increasing research efforts, *E. milii* is expected to become a key plant species for green nanotechnology, offering an environmentally friendly approach to nanoparticle synthesis with wide-ranging applications.

2.2 Taxonomy and Botanical Description of *Euphorbia milii*

E. milii is a flowering plant native to Madagascar that belongs to the spurge family Euphorbiaceae. *E. milii* is a woody shrub that can grow up to three feet tall, though hybrids vary in height. The splendens variety, in particular, can reach up to 5 to 6 feet. Its branching, greyish-brown stems, which have 5 to 7 sides, are well-suited for water storage. While some clones have few or no thorns, most plants feature sharp, one-inch grey spines along their stems and branches.

2.2.1 Taxonomical classification

E. milii is widely distributed in tropical and subtropical regions and is renowned for its medicinal properties. Its taxonomy is as follows: Kingdom: Plantae, Phylum: Angiosperms, Class: Eudicots, Order: Malpighiales, Family: Euphorbiaceae, Genus: Euphorbia and Species: Euphorbia milii. The plant is known for its bioactive secondary metabolites, including flavonoids, alkaloids, terpenoids, and phenolics, which contribute to its pharmacological effects (Mutalib et al. 2020). Traditional uses of *E. milii* include antimicrobial, anti-inflammatory, and wound-healing treatments, indicating its potential as a source of natural bioactive compounds for various applications, including nanoparticle synthesis (Bawazeer 2024).

Euphorbiaceae Family

The Euphorbiaceae, commonly referred to as the spurge family, is a diverse group of flowering plants encompassing over 300 genera and approximately 8,000 species (Benjamaa et al. 2022). Characterized by simple, alternate leaves and often containing milky sap, members of this family thrive in both temperate and tropical regions (Althobaiti 2023). Notable species include the poinsettia (*Euphorbia pulcherrima*), cassava (*Manihot esculenta*), and rubber tree (*Hevea brasiliensis*). While many species are valued for their ornamental or medicinal properties, others hold significant economic importance, producing resources such as rubber, oil, and starch. However, some members of the Euphorbiaceae pose risks to human and animal health due to the presence of toxic compounds (Reback et al. 2021).

***Euphorbia* Genus**

The *Euphorbia* genus, the largest and most diverse within the Euphorbiaceae family, encompasses over 2,000 species distributed across temperate, tropical, and arid regions worldwide (Shaker et al. 2022). Known for their remarkable adaptability, these plants exhibit a wide range of growth forms, including herbaceous plants, shrubs, and trees, as well as succulents that mimic cacti in appearance due to convergent evolution. A defining characteristic of *Euphorbia* species is their unique inflorescence structure, called a cyathium, which mimics a single flower but is actually a cluster of reduced flowers surrounded by bracts (Prenner & Rudall 2007). Many species produce a toxic, milky latex that serves as a defence mechanism against herbivores and pathogens, though this sap can cause irritation or poisoning in humans and animals. Economically and culturally significant, *Euphorbia* includes species prized for ornamental purposes, such as the poinsettia (*Euphorbia pulcherrima*), and others valued for medicinal, industrial, or ecological roles (Salehi et al. 2019). However, the genus also includes invasive species that pose ecological challenges in some regions.

***Euphorbia milii* Species**

E. milii, is a woody, spiny, succulent shrub native to Madagascar, renowned for its vibrant red, pink, or yellow flowers and its resilience in adverse conditions showed in Figure 2.2 (Althobaiti 2023). Beyond its ornamental appeal, this species has been traditionally utilized for its medicinal properties. Flavonoids and terpenoids, which are naturally present in abundance in the leaves and stems, have been found to possess antibacterial, antioxidant, and anti-inflammatory properties (Mutalib et al., 2023). Because of these benefits, *E. milii* shows great potential for green nanotechnology, particularly in the eco-friendly production of nanoparticles for biomedical applications.



Figure 2.2 Diagram of inflorescence (various flower parts) of flowering species *E. milii* (Family Euphorbiaceae) (Althobaiti 2023)

2.2.2 Morphological characteristics

Agarwal and Prakash (2024) described *E. milii* as a hardy succulent with thick, fleshy stems covered in sharp thorns. These thorns serve as a natural defence, helping the plant resist herbivores. Its leaves are usually small, oval-shaped, and grow alternately along the stems. Depending on the cultivar, the leaves can vary in colour and are often deciduous, meaning they may shed over time.

One of the most striking features of *E. milii* is its bright, petal-like bracts, which are often mistaken for flowers. The actual flowers, however, are tiny and barely noticeable, nestled in the centre of these colourful bracts. The plant's ornamental appeal is further enhanced by the wide range of bract colours, including red, pink, yellow, and orange (Suleiman et al., 2020). These flowers grow in small, compact clusters known as cymes at the tips of the stems, with the vibrant bracts surrounding them, making the plant even more visually appealing.

Additionally, *E. milii* also generates a milky latex that is found throughout the plant. Because of its toxicity and potential to irritate skin when touched, this latex must be handled carefully (Ahmad et al. 2019).

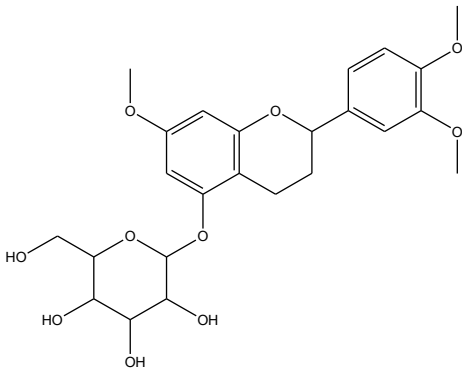
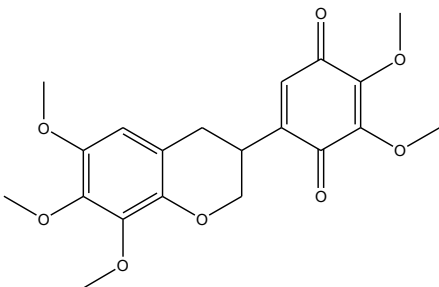
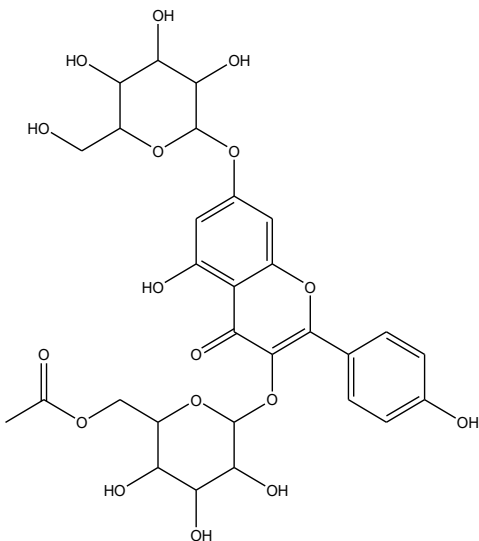
2.3 Phytochemical Composition of *Euphorbia milii*

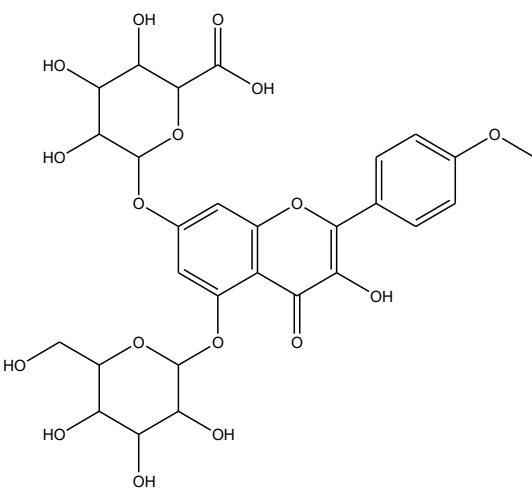
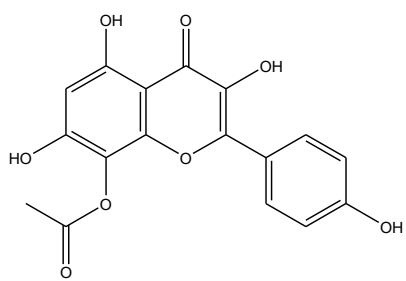
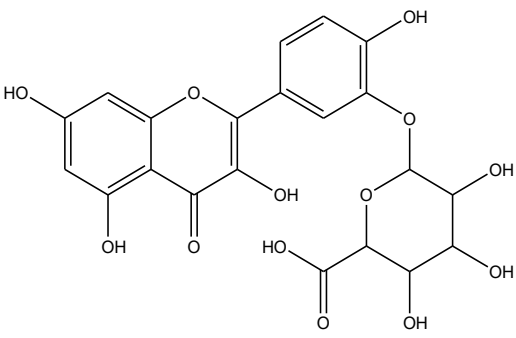
The phytochemical composition of *E. milii* plays a crucial role in its biological activities and potential applications. This plant, belonging to the Euphorbiaceae family, is known to contain a diverse range of bioactive compounds, including flavonoids, alkaloids, tannins, phenolics, terpenoids, and saponins. These secondary metabolites contribute to its antioxidant, antimicrobial, and medicinal properties (Negm et al. 2024). Among them, flavonoids such as quercetin, rutin, and vitexin are of particular interest due to their ability to act as natural reducing and stabilizing agents in green nanoparticle synthesis. Understanding the phytochemical profile of *E. milii* is essential for exploring its potential in biomedical and nanotechnological applications.

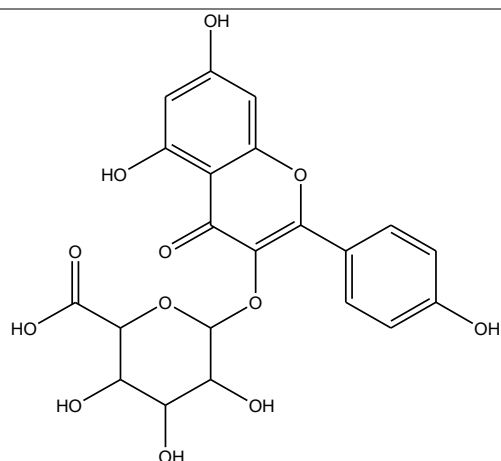
2.3.1 Identification of key bioactive compounds in *E. milii*

The identification of key bioactive compounds in *E. milii* is essential for understanding its pharmacological potential and its role in green synthesis applications. Its bioactive components are frequently characterized and quantified using a variety of analytical methods, including as nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC).. Among the major bioactive compounds found in *E. milii*, flavonoids, phenolic acids, alkaloids, terpenoids, and tannins have been identified (Saleem et al. 2019). These compounds exhibit strong antioxidant, anti-inflammatory, and antimicrobial properties, making them valuable for medicinal and nanotechnological applications. In particular, flavonoids play a critical role as natural reducing and stabilizing agents in the green synthesis of silver nanoparticles (AgNPs), enhancing their stability and biocompatibility. Further phytochemical profiling and structural elucidation of these compounds can provide deeper insights into their biological activities and potential therapeutic uses. Table 2.1 shows a few flavonoid compounds found by the other researchers.

Table 2.1 Flavonoid compounds found by the other researchers

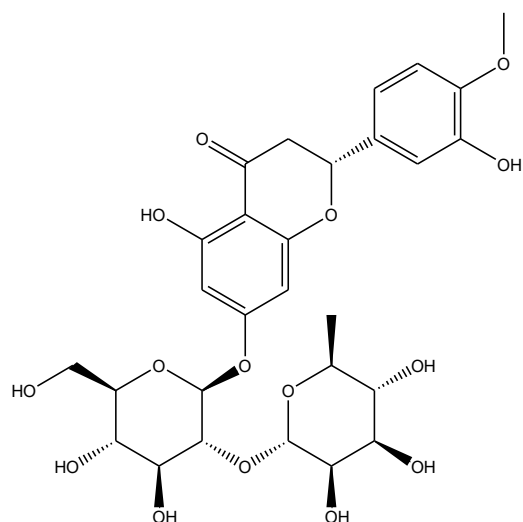
<i>E. milii</i> Part	Flavonoid Identified	Compound Structure	Reference s
<i>E. milli</i> root methanol extract	Dichotosinin		Saleem et al. (2019)
	Abruquinone B		Saleem et al. (2019)
	Kaempferol 3-(6''-acetylglucoside)-7-glucoside		Saleem et al. (2019)

	Kaempferide 5-glucoside-7-glucuronid 	Saleem et al. (2019)
	Herbacetin 8-acetate 	Saleem et al. (2019)
E. coli aerial ethyl acetate fraction	Quercetin-3-glucuronide 	Hassan et al. (2023)
	Kaempferol-3-glucuronide	Hassan et al. (2023)



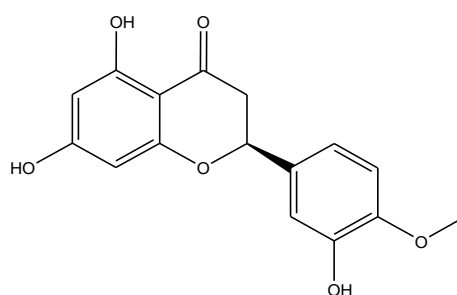
Hesperetin-7-
O-
neohesperidos
ide

Hassan et
al. (2023)



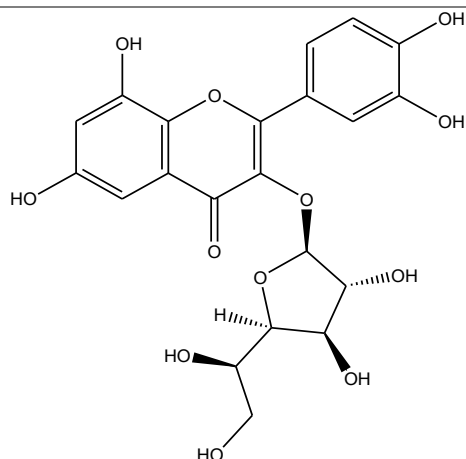
Hesperetin

Hassan et
al. (2023)

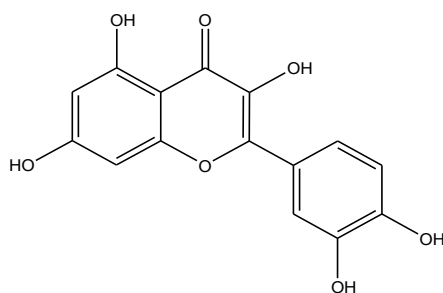


Isoquercitrin

Hassan et
al. (2023)

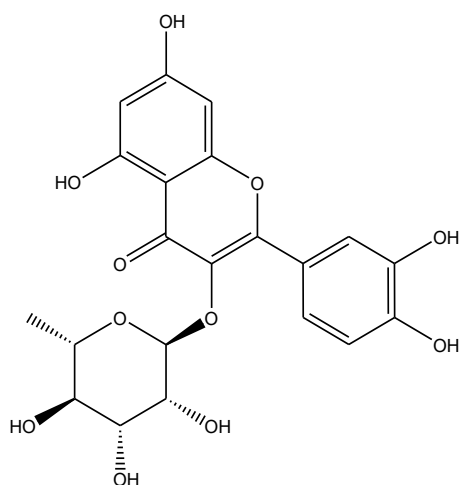


Quercetin



Ekeke &
Ndukwu
(2014);
Hassan et
al. (2023)

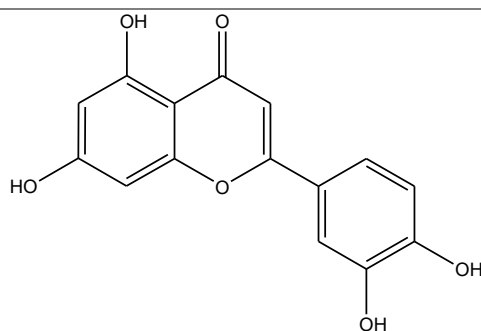
Quercitrin



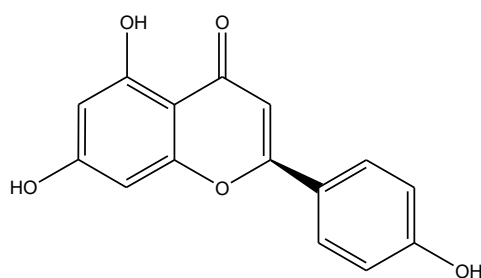
Hassan et
al. (2023)

Luteolin

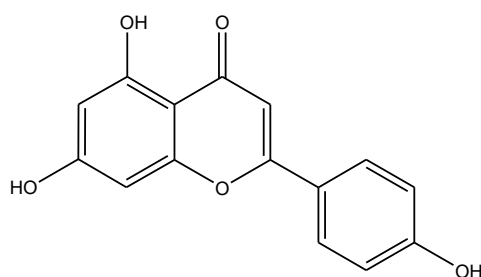
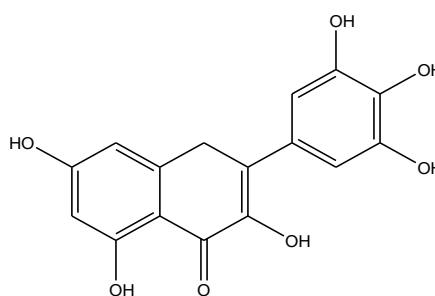
Hassan et
al. (2023)



Naringenin

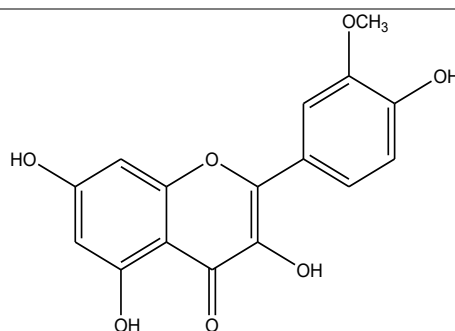
Hassan et
al. (2023)

Apigenin

Ekeke &
Ndukwu
(2014);
Hassan et
al. (2023)**Whole
Part** MyricetinEkeke &
Ndukwu
(2014)

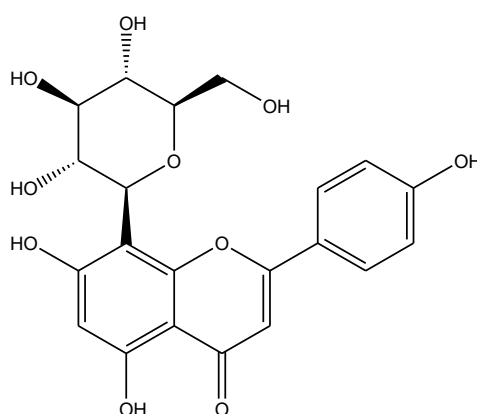
Isorhamnetin

Ekeke &
Ndukwu
(2014)



Vitexin

Ekeke &
Ndukwu
(2014)



2.3.2 Role of flavonoids, terpenoids, and other secondary metabolites in medicinal applications

Extensive research on *E. milii* has identified a rich diversity of secondary metabolites contributing to its therapeutic properties. Notably, the plant contains flavonoids, terpenoids, alkaloids, phenolic compounds, and tannins, each playing a significant role in its medicinal applications (Roy et al. 2022).

Flavonoids: Compounds such as kaempferol and quercetin, extracted from *E. milii*, exhibit potent anti-inflammatory and antioxidant activities. These properties are instrumental in mitigating oxidative stress and inflammation-related disorders (Reza et al. 2023). **Terpenoids:** Abundant in *E. milii*, terpenoids possess notable pharmacological effects, including anti-inflammatory, anticancer, and antibacterial activities. These compounds contribute to the plant's efficacy in traditional medicine

for treating various ailments. (Saleem et al. 2019). Phenolic Compounds: The presence of phenolic compounds, such as tannins, in *E. milii* imparts significant antioxidant activity, aiding in the scavenging of free radicals and offering protective effects against cellular damage (Dai & Mumper 2010).

Historically, *E. milii* has been utilized in traditional medicine to address a range of conditions, including skin infections, warts, cancer, hepatic disorders, and inflammatory diseases (Salehi et al. 2019). The therapeutic efficacy is attributed to the presence of bioactive compounds in its latex and leaf extracts. These findings underscore the potential of *E. milii* as a valuable source of bioactive compounds for pharmaceutical applications. Further studies are warranted to explore and validate its therapeutic potential in modern medicine.

2.4 Flavonoids as Reducing and Stabilizing Agents in Nanoparticle Synthesis

Flavonoids, a broad class of polyphenolic compounds widely distributed in plants, play a crucial role in the environmentally friendly synthesis of nanoparticles. Their strong antioxidant properties enable them to donate electrons, thereby reducing metal ions such as Ag^+ to their metallic nanoparticle form (Mittal et al., 2013). The production of nanoparticles depends on this reduction process because it guarantees that ionic metal precursors will be transformed into stable structures at the nanoscale. Plant extracts containing various phytochemicals that contribute to the formation of NPs are depicted in Figure 2.3. Flavonoid concentration, pH, and temperature all affect how well flavonoid-mediated synthesis works. These variables can be adjusted to regulate the size and shape of the nanoparticles.

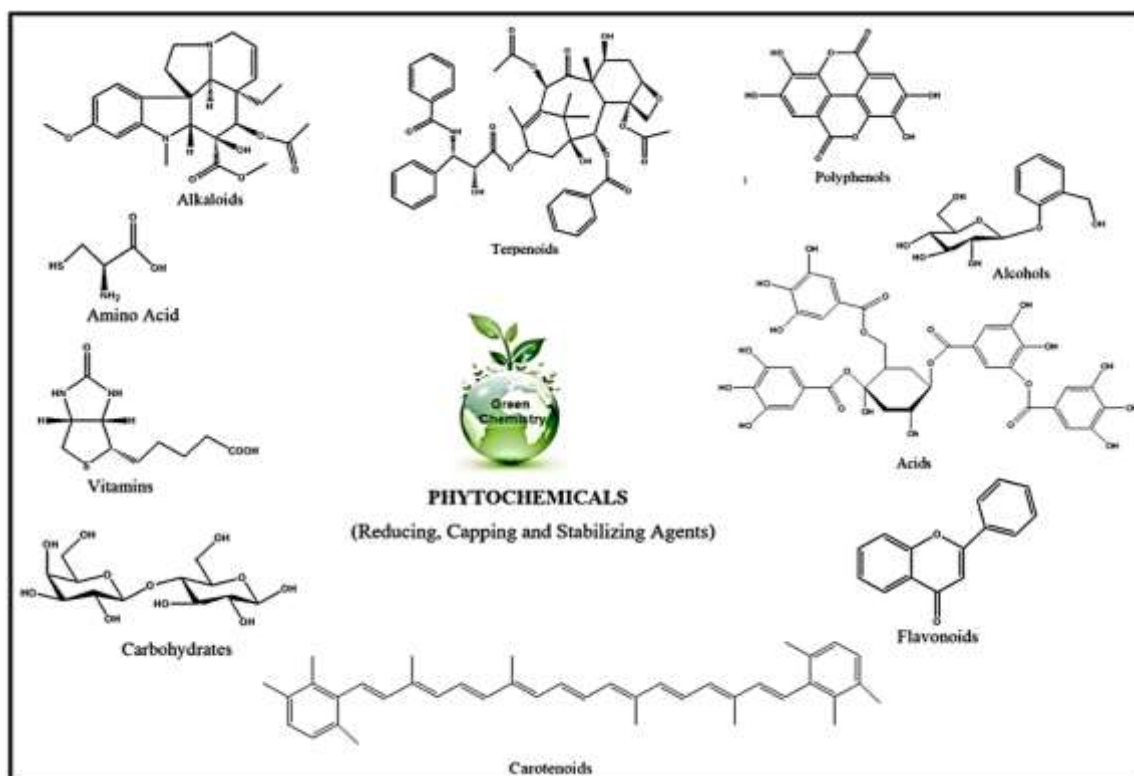


Figure 2.3 Illustration of several phytochemicals found in plant extracts that contribute to the formation of NPs (Rani et al. 2023)

Beyond their role as reducing agents, flavonoids act as natural stabilizers in nanoparticle synthesis. Due to their multiple hydroxyl and carbonyl groups, flavonoids exhibit strong binding affinity to the nanoparticle surface, forming a protective capping layer that prevents aggregation and enhances colloidal stability (Singh et al. 2018). This stabilization is crucial for maintaining nanoparticle dispersibility in aqueous solutions, ensuring their longevity and effectiveness in various applications. Additionally, flavonoid-capped nanoparticles exhibit improved biocompatibility, making them highly suitable for biomedical use, including drug delivery and antimicrobial therapies.

The functional properties of flavonoid-stabilized nanoparticles extend beyond synthesis and stability, as these natural compounds impart additional biological activities. Studies have shown that flavonoid-coated nanoparticles exhibit enhanced antimicrobial, antioxidant, and anticancer properties compared to their chemically synthesized counterparts (Singh et al. 2018). The synergistic interaction between flavonoids and nanoparticles contributes to improved therapeutic outcomes, making them attractive for applications in nanomedicine. As research continues, flavonoid-mediated nanoparticle synthesis holds significant potential for advancing eco-friendly nanotechnology and biomedical innovations.

2.4.1 Chemical Structure and Classification of Flavonoids

Flavonoids are a diverse group of polyphenolic compounds widely distributed in plants, including *E. milii*. They are characterized by a 15-carbon skeleton (C6-C3-C6) composed of two aromatic rings (A and B) linked by a three-carbon bridge forming the heterocyclic C-ring (Dias et al. 2021). The structural diversity of flavonoids arises from variations in hydroxylation, methylation, glycosylation, and other substitutions, influencing their biological activity and solubility. Based on structural differences, flavonoids are classified into several subgroups, including flavonols (e.g., quercetin, rutin), flavones (e.g., vitexin, luteolin), flavanones, flavanols, isoflavones, and anthocyanins (Šamec et al. 2021). These compounds play significant roles in antioxidant, anti-inflammatory, and antimicrobial activities, as well as in the green synthesis of nanoparticles, where they act as natural reducing and stabilizing agents. Understanding the chemical structure and classification of flavonoids is essential for exploring their potential in biological and nanotechnological applications. Figure 2.4 Representative structures of major classes of flavonoids.

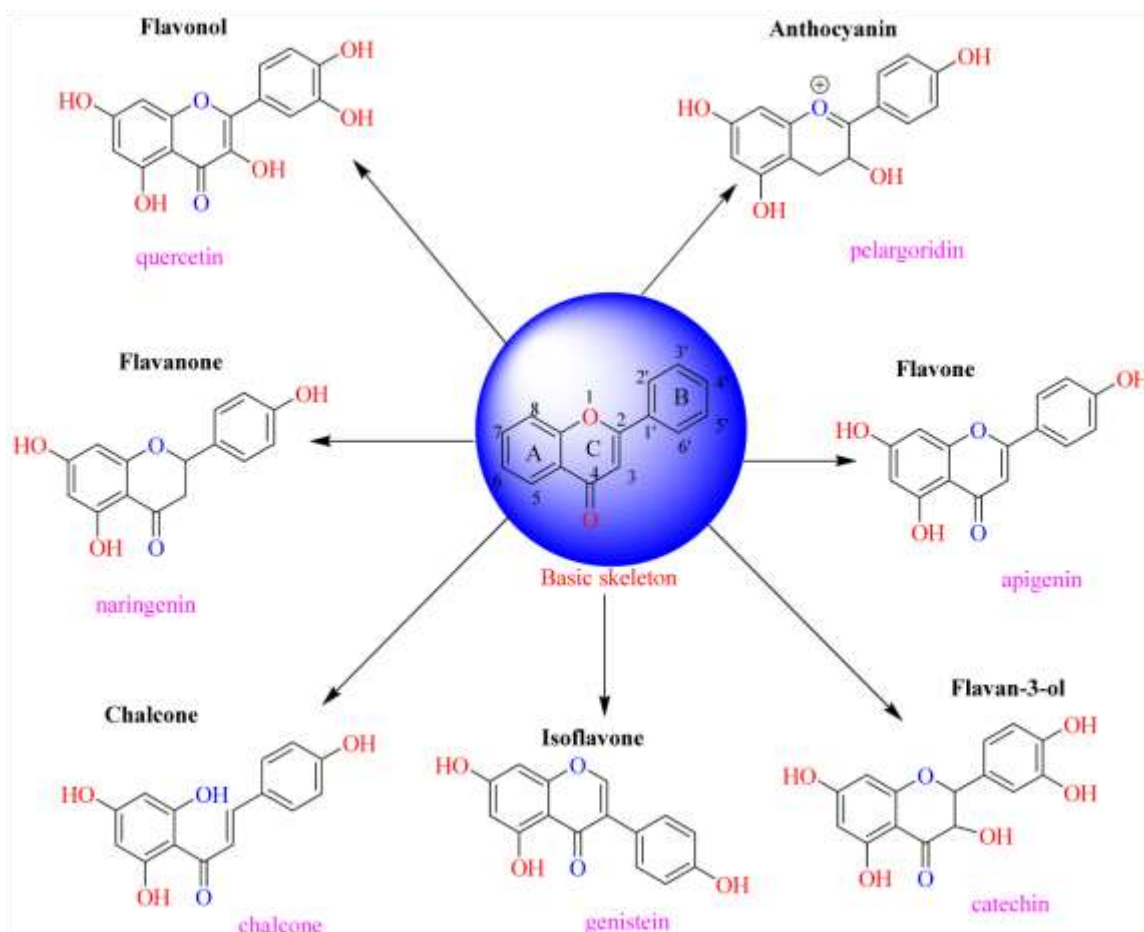


Figure 2.4 Representative structures of major classes of flavonoids (Magozwi et al. 2021)

2.4.2 Mechanism of flavonoid-mediated nanoparticle synthesis

Flavonoid-mediated nanoparticle synthesis is a green and sustainable approach that utilizes plant-derived flavonoid compounds as natural reducing and stabilizing agents. The mechanism involves three key stages: reduction of metal ions, nucleation, and stabilization of nanoparticles. In this process, flavonoids donate electrons to reduce silver ions (Ag^+) to elemental silver (Ag^0), initiating nanoparticle formation (Hou et al. 2023). The presence of hydroxyl ($-\text{OH}$) and carbonyl ($-\text{C}=\text{O}$) functional groups in flavonoids enhances their reducing ability, accelerating the conversion of metal salts into stable nanoparticles.

Following reduction, the nucleation phase begins, where reduced silver atoms aggregate to form small nanoparticle seeds. These seeds further grow into uniformly

distributed nanoparticles, influenced by the concentration of flavonoids and reaction conditions such as pH, temperature, and incubation time. Finally, flavonoids act as capping agents, surrounding the nanoparticles and preventing excessive aggregation, thereby enhancing their stability and biocompatibility (Ying et al. 2022). The presence of specific flavonoids, such as quercetin, rutin, and vitexin, plays a crucial role in determining the size, morphology, and stability of the synthesized nanoparticles. Understanding this mechanism provides valuable insights into optimizing flavonoid-assisted green synthesis for biomedical and nanotechnological applications. Figure 2.5 showed mechanism of reduction of silver ions to AgNPs by quercetin molecule

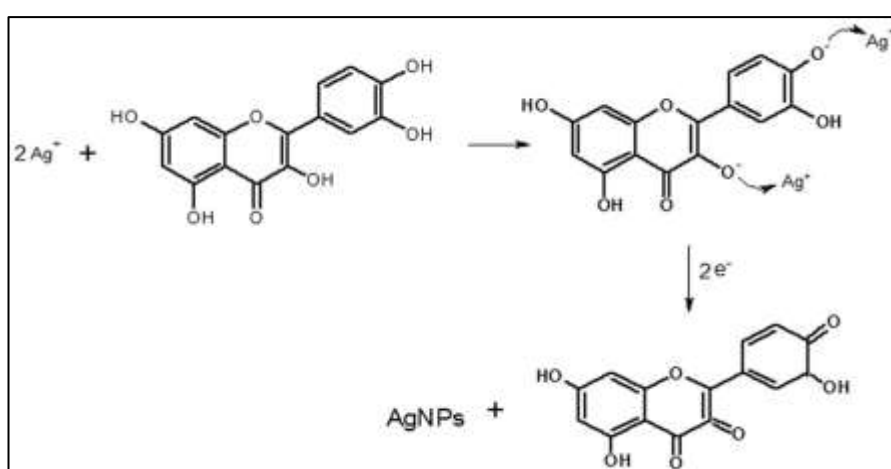


Figure 2.5 Mechanism of quercetin molecule reduces silver ions to AgNPs (Jain & Mehata 2017)

2.4.3 Advantages of Flavonoid-Based AgNPs over Chemically Synthesized AgNPs

Flavonoid-based silver nanoparticles (AgNPs) have several advantages over chemically synthesized AgNPs, particularly in terms of biocompatibility and reduced toxicity. Traditional chemical synthesis of AgNPs often involves toxic reducing agents such as sodium borohydride, which can lead to harmful residues affecting biomedical applications (Ahmed et al. 2016). In contrast, flavonoid-based AgNPs utilize natural plant-derived compounds that not only reduce silver ions but also provide additional therapeutic benefits, such as antimicrobial and antioxidant properties. This

biocompatibility is especially advantageous for applications in drug delivery, wound healing, and cancer treatment.

Another significant advantage of flavonoid-mediated synthesis is the enhanced stability and dispersibility of the nanoparticles. Flavonoids act as capping agents, preventing particle aggregation and oxidation, thereby increasing their shelf life and effectiveness (Rao et al. 2021). Chemically synthesized AgNPs often require synthetic stabilizers, which can be costly and may introduce unwanted toxicity. The inherent stabilizing capability of flavonoids makes the green synthesis approach not only safer but also more cost-effective and scalable for industrial production.

Furthermore, flavonoid-functionalized AgNPs demonstrate superior biological activity. Studies have shown that the presence of flavonoids enhances the antimicrobial and anticancer effects of AgNPs by promoting oxidative stress and apoptosis in target cells (Sharma et al. 2020). The combination of silver nanoparticles with flavonoids creates a synergistic effect, improving the therapeutic potential of these nanomaterials. Given these benefits, flavonoid-based AgNPs are increasingly being explored as a safer and more effective alternative to chemically synthesized nanoparticles in medical and environmental applications.

2.5 Green Synthesis of Silver Nanoparticles (AgNPs) Using Plant Extracts

The eco-friendly synthesis of AgNPs using plant extracts has gained significant attention due to its promising biomedical applications and minimal environmental impact. This method relies on plant-derived biomolecules like flavonoids, tannins, terpenoids, and polyphenols, which act as both reducing and stabilizing agents to convert silver ions (Ag^+) into nanoparticles (Mittal et al., 2013). Unlike conventional chemical synthesis, which requires hazardous reagents, green synthesis eliminates toxic byproducts, making it more suitable for pharmaceutical and biomedical use (Ahmed et al. 2016).

One of the key advantages of using plant extracts for nanoparticle synthesis is their ability to control the size and shape of AgNPs through optimization of reaction

parameters, such as pH, temperature, and concentration of plant metabolites. Studies have shown that varying these parameters can result in AgNPs with different morphological characteristics, which directly influence their biological properties (Singh et al. 2018). Additionally, plant-mediated synthesis ensures better dispersibility and stability of nanoparticles due to the presence of natural capping agents that prevent agglomeration.

Furthermore, green-synthesized AgNPs exhibit strong antibacterial, antifungal, and anticancer properties due to their interaction with microbial and cancerous cells. The bioactive compounds present in plant extracts enhance the cytotoxicity of AgNPs against various pathogens and cancer cell lines, making them potential candidates for drug delivery and therapeutic applications (Sharma et al. 2020). As research advances, the green synthesis of AgNPs is expected to become a mainstream technique for nanomedicine, offering a safer and more effective alternative to conventional nanoparticles.

2.5.1 Principles of Green Synthesis and its Environmental Benefits

Green synthesis refers to the process of using natural materials, such as plant extracts, to convert metal ions into nanoparticles. This eco-friendly approach avoids harmful chemicals and high energy consumption, making it a safer alternative to conventional methods. It's also simple, cost-effective, and scalable, which adds to its appeal. AlHarbi & Felimban (2023) illustrated the plant-based synthesis of AgNPs in Figure 2.6.

One of the major environmental benefits of green synthesis is the elimination of toxic chemicals such as sodium borohydride, hydrazine, and organic solvents, which are commonly used in conventional nanoparticle synthesis (Fahim et al. 2024). This significantly reduces chemical waste and pollution, making the process safer for both researchers and the environment.

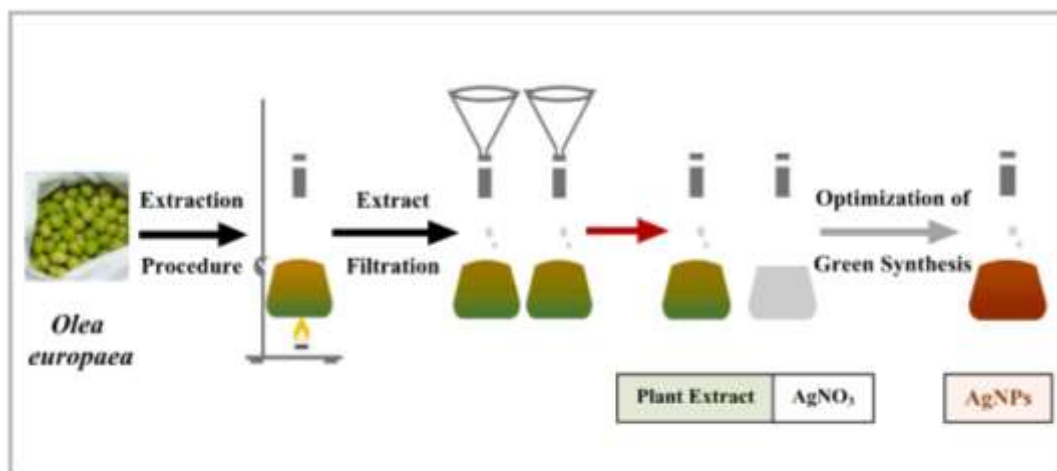


Figure 2.6 Plant-based synthesis of AgNPs (AlHarbi & Felimban 2023)

Furthermore, using plant-based extracts ensures that the synthesis process is cost-effective, scalable, and biocompatible, making it a great fit for applications in medicine, environmental cleanup, and pharmaceuticals (Hou et al., 2023). Since the synthesized nanoparticles exhibit enhanced biocompatibility and reduced toxicity, they are ideal for drug delivery, antimicrobial treatments, and other medical applications.

Overall, the principles of green synthesis align with the goals of sustainable development and green chemistry, promoting an environmentally friendly alternative to traditional nanoparticle synthesis methods while ensuring efficiency, safety, and economic feasibility.

2.5.2 Role of *Euphorbia milii* in the green synthesis of AgNPs

The plant *E. milii* has emerged as a promising candidate for the green synthesis of silver nanoparticles (AgNPs) due to its rich phytochemical composition. This plant contains bioactive compounds such as flavonoids, tannins, alkaloids, and terpenoids, which act as natural reducing and stabilizing agents in nanoparticle synthesis (Hasan et al. 2020). Table 2.2 shows the summary of qualitative phytochemical analysis tests on crude extract of *E. milii*. The ability of these compounds to donate electrons facilitates the reduction of silver ions (Ag^+) to metallic silver (Ag^0), leading to the formation of AgNPs with enhanced stability and functionality. The use of *E. milii* not

only eliminates the need for toxic reducing agents but also imparts additional bioactive properties to the synthesized nanoparticles.

Table 2.2 An overview of the qualitative phytochemical analysis studies conducted on *E. milii* crude extracts (Mutalib et al. 2020)

Phytochemical test	Hexane		Ethyl acetate		Methanol	
	Flower	Leaves	Flower	Leaves	Flower	Leaves
Alkaloid Test	+	+	+	+	+	+
Triterpenoid Test	+	+	+	–	–	–
Steroid Test	–	–	–	+	+	+
Flavonoid Test	+	+	+	+	+	+
Quinones Test	+	+	+	+	+	+
Phlobatannins Test	–	–	–	–	–	–
Saponins Test	–	–	–	+	–	–
Reducing Sugar Test	+	+	+	+	+	+
Carbohydrate Test	+	+	+	+	+	+
Phenolic Content Test	–	–	+	+	+	+
Tannin test	–	–	+	+	+	+

Studies have shown that AgNPs synthesized using *E. milii* extracts exhibit superior physicochemical properties, including controlled particle size, high stability, and strong antibacterial and anticancer activities (Rajakumar et al. 2017). The synthesis process typically involves mixing an aqueous extract of *E. milii* with a silver nitrate (AgNO_3) solution, followed by incubation under optimal conditions. The resulting nanoparticles are characterized using techniques such as UV-Vis spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM) to confirm their formation and stability.

Beyond biomedical applications, AgNPs derived from *E. milii* have demonstrated potential in environmental remediation. Due to their high surface area and reactivity, these nanoparticles are effective in water purification, heavy metal removal, and the degradation of toxic organic pollutants (Subramani et al. 2018). The integration of *E. milii* in nanotechnology aligns with sustainable and cost-effective

approaches, making it a valuable resource for large-scale production of AgNPs. As research in this field progresses, *E. milii*-mediated AgNPs are expected to play a crucial role in green nanotechnology, offering eco-friendly alternatives for various industrial and biomedical applications.

2.6 Cytotoxicity of Silver Nanoparticles (AgNPs) in Cancer Treatment

Silver nanoparticles (AgNPs) have emerged as promising agents in cancer treatment due to their unique cytotoxic effects on cancer cells. Their ability to induce oxidative stress, disrupt cellular functions, and trigger apoptosis has made them an attractive alternative to conventional chemotherapeutic agents (Gurunathan et al. 2019). Unlike traditional cancer treatments that often harm both cancerous and healthy cells, AgNPs exhibit selective toxicity toward malignant cells while causing minimal damage to normal tissues. This selectivity is largely attributed to differences in cellular metabolism and membrane composition between healthy and cancerous cells.

The production of reactive oxygen species (ROS) is one of the main ways that AgNPs cause harm to cells. Overproduction of ROS can result in oxidative stress, which can harm DNA, malfunction mitochondria, and trigger apoptotic pathways (Zhang et al. 2020). Additionally, AgNPs can directly interact with cellular proteins and disrupt key signaling pathways involved in cell survival and proliferation. Studies have shown that AgNPs effectively inhibit the growth of various cancer cell lines, including breast cancer (MCF-7), lung cancer (A549), and cervical cancer (HeLa), highlighting their broad-spectrum anticancer potential.

Moreover, AgNPs have demonstrated synergistic effects when combined with natural bioactive compounds such as flavonoids and polyphenols. These combinations enhance the therapeutic efficacy of AgNPs by improving cellular uptake and increasing oxidative stress in cancer cells (Singh et al., 2018). However, despite their promising anticancer potential, it remains crucial to thoroughly evaluate the long-term biocompatibility of AgNPs and their potential cytotoxic effects on healthy cells. Ongoing research therefore aims to optimize AgNP formulations to maximize their

therapeutic index while minimizing adverse side effects, paving the way for potential clinical applications.

2.6.1 Mechanism of AgNP-induced cytotoxicity

The cytotoxicity of silver nanoparticles (AgNPs) in cancer treatment is primarily attributed to their ability to generate oxidative stress, induce mitochondrial damage, and trigger apoptosis. One of the key mechanisms by which AgNPs exert their anticancer effects is through the production of reactive oxygen species (ROS) (Xu et al. 2020). Excess ROS accumulation leads to oxidative damage of cellular components, including proteins, lipids, and DNA, which ultimately disrupts normal cellular functions and promotes cell death. Cancer cells, which already exhibit elevated oxidative stress levels compared to normal cells, are particularly vulnerable to further ROS-induced damage.

Mitochondrial dysfunction is another major consequence of AgNP-induced cytotoxicity. AgNPs disrupt mitochondrial membrane potential, impair ATP production, and lead to the release of pro-apoptotic factors such as cytochrome c (Gurunathan et al. 2018). These events activate caspase-mediated apoptotic pathways, resulting in programmed cell death. Additionally, AgNPs interfere with key cellular signaling pathways involved in cell survival and proliferation, including the PI3K/Akt and MAPK pathways, further sensitizing cancer cells to apoptosis (Ebrahimzadeh et al. 2021).

Beyond apoptosis, AgNPs can also induce autophagy, a cellular self-degradation process that may either promote or inhibit cancer cell survival. While some studies suggest that AgNP-induced autophagy enhances cancer cell death, others indicate that autophagy serves as a protective mechanism against nanoparticle toxicity (Manke et al. 2021). Understanding the precise balance between these mechanisms is crucial for optimizing AgNP-based cancer therapies. Future research aims to fine-tune AgNP formulations to enhance their selective toxicity toward cancer cells while minimizing adverse effects on normal tissues.

2.6.2 Comparison of Free Flavonoids vs. Flavonoid-Conjugated AgNPs in Cytotoxic Activity

Flavonoids are well-known for their antioxidant and anticancer properties, but their therapeutic potential is often limited by poor bioavailability and rapid metabolism in the body. Free flavonoids, when administered alone, exhibit moderate cytotoxic effects against cancer cells, primarily through oxidative stress modulation and apoptosis induction (Kumar & Pandey 2013). However, their low solubility and instability in physiological conditions reduce their effectiveness. To overcome these limitations, researchers have explored the conjugation of flavonoids with silver nanoparticles (AgNPs) to enhance their biological activity.

Flavonoid-conjugated AgNPs exhibit superior cytotoxicity compared to free flavonoids due to improved cellular uptake and prolonged retention in tumor microenvironments (Singh et al. 2018). The conjugation process enhances the stability of flavonoids, allowing for sustained release and increased bioavailability. Additionally, the presence of AgNPs amplifies the anticancer effects by inducing reactive oxygen species (ROS) generation and disrupting mitochondrial function. This synergistic interaction significantly boosts the therapeutic efficacy of flavonoids while reducing the required dosage, thereby minimizing potential side effects.

Furthermore, flavonoid-functionalized AgNPs demonstrate enhanced selectivity toward cancer cells compared to free flavonoids. Due to the unique physicochemical properties of nanoparticles, flavonoid-coated AgNPs preferentially accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect (Yallapu et al. 2021). This targeted action minimizes toxicity to healthy cells and improves treatment outcomes. Given these advantages, flavonoid-conjugated AgNPs hold great promise for future cancer therapies, warranting further clinical investigation and optimization for safe and effective use.

2.7 Previous Studies on Cytotoxicity of Flavonoid-Based Silver Nanoparticles

Several studies have investigated the cytotoxic effects of flavonoid-based silver nanoparticles (AgNPs) against various cancer cell lines, demonstrating their potential as effective anticancer agents. Flavonoids, such as quercetin, kaempferol, and catechin, have been widely studied for their ability to enhance the cytotoxicity of AgNPs by improving cellular uptake and promoting reactive oxygen species (ROS) generation (Mittal et al. 2013). These bioactive compounds act as both reducing and stabilizing agents in AgNP synthesis, ensuring nanoparticle stability while simultaneously providing additional therapeutic effects.

Recent research has shown that flavonoid-coated AgNPs exhibit significantly higher anticancer activity compared to free flavonoids or chemically synthesized AgNPs. A study by Sharma et al. (2020) reported that quercetin-functionalized AgNPs induced apoptosis in MCF-7 breast cancer cells more effectively than quercetin alone. The study suggested that the combination of flavonoids with AgNPs enhances mitochondrial dysfunction and caspase activation, leading to increased cancer cell death. Another study by Zhang et al. (2019) found that flavonoid-coated AgNPs demonstrated selective cytotoxicity against lung cancer (A549) and cervical cancer (HeLa) cells while exhibiting minimal toxicity toward normal fibroblast cells.

Moreover, flavonoid-based AgNPs have shown potential in overcoming multidrug resistance (MDR) in cancer cells. Some cancer cells develop resistance to conventional chemotherapy drugs through efflux pump mechanisms, but studies indicate that AgNPs conjugated with flavonoids can inhibit these pumps, thereby restoring drug sensitivity (Singh et al. 2018). This ability to enhance drug efficacy while minimizing side effects makes flavonoid-mediated AgNPs a promising avenue for future anticancer treatments. As research continues, the development of optimized flavonoid-AgNP formulations with enhanced stability, bioavailability, and targeted action remains a key focus in nanomedicine.

2.7.1 Review of Recent Studies on Flavonoid-Mediated AgNP Cytotoxicity

Flavonoid-mediated silver nanoparticles (AgNPs) have been extensively studied for their cytotoxic effects against various cancer cell lines. Recent studies have demonstrated that flavonoid-coated AgNPs possess enhanced anticancer properties compared to free flavonoids or conventional AgNPs due to their improved cellular uptake, prolonged retention, and increased bioavailability (Das et al. 2020). These nanoparticles induce cytotoxicity primarily through oxidative stress, apoptosis, and disruption of key cellular pathways involved in cancer progression. The combination of flavonoids with AgNPs enhances the therapeutic potential of these nanoparticles, making them promising candidates for targeted cancer therapy.

Khan et al. (2021) examined the cytotoxic effects of kaempferol-functionalized AgNPs on MCF-7 breast cancer cells and discovered that they caused apoptosis by caspase activation and mitochondrial damage, which dramatically decreased cell viability. Similarly, by producing reactive oxygen species (ROS) and inducing cell cycle arrest, quercetin-conjugated AgNPs successfully suppressed the growth of cervical cancer (HeLa) and lung cancer (A549) cells (Raj et al., 2019). These investigations demonstrate how flavonoids help AgNPs' anticancer effectiveness while preserving their biocompatibility with healthy cells.

Furthermore, recent research has explored the synergistic effects of flavonoid-mediated AgNPs in combination with conventional chemotherapy drugs. A study by Prasad et al. (2022) demonstrated that flavonoid-based AgNPs enhanced the sensitivity of multidrug-resistant cancer cells to doxorubicin, a commonly used chemotherapeutic agent. The study suggested that flavonoid-coated AgNPs inhibit drug efflux pumps, thereby increasing intracellular drug accumulation and improving treatment outcomes. These findings indicate that flavonoid-mediated AgNPs could serve as effective adjuvants in cancer therapy, potentially reducing drug resistance and side effects associated with chemotherapy.

2.7.2 Effect of AgNPs on Different Cancer Cell Lines

Silver nanoparticles (AgNPs) have shown promising cytotoxic effects against various cancer cell lines, making them potential candidates for anticancer therapies. The cytotoxicity of AgNPs varies depending on the type of cancer cell, particle size, surface charge, and functionalization with bioactive compounds such as flavonoids (Gurunathan et al. 2019). Studies have demonstrated that AgNPs effectively inhibit the proliferation of multiple cancer cell lines, including breast cancer (MCF-7), lung cancer (A549), and cervical cancer (HeLa), primarily through oxidative stress generation, mitochondrial dysfunction, and apoptosis induction.

In breast cancer cells (MCF-7), AgNPs have been reported to induce cell cycle arrest and apoptosis by activating the caspase cascade and downregulating anti-apoptotic proteins such as Bcl-2 (J. Zhang et al. 2021). Similarly, in lung cancer cells (A549), AgNPs trigger reactive oxygen species (ROS) accumulation, leading to DNA damage and mitochondrial membrane depolarization (Patra et al. 2020). The selective cytotoxicity of AgNPs in cancer cells is largely attributed to their enhanced uptake and intracellular accumulation, which disrupts cellular homeostasis and promotes programmed cell death while minimizing toxicity to normal cells.

In cervical cancer cells (HeLa), studies have shown that flavonoid-coated AgNPs exhibit stronger cytotoxic effects than chemically synthesized AgNPs. This is due to the ability of flavonoids to enhance nanoparticle stability, bioavailability, and cellular uptake, leading to increased oxidative stress and apoptotic signaling (Maiti et al. 2022). The combination of AgNPs with flavonoids results in a synergistic effect, improving their anticancer potential while reducing side effects associated with conventional chemotherapy. Future research is focused on optimizing nanoparticle formulations to enhance targeted delivery and minimize off-target toxicity, paving the way for AgNP-based nanomedicine.

2.7.3 Potential biomedical applications and limitations

Silver nanoparticles (AgNPs) have demonstrated significant potential in biomedical applications due to their strong antimicrobial, anticancer, and anti-inflammatory properties. In nanomedicine, AgNPs are widely explored for drug delivery systems, wound healing, and biosensors (Rai et al. 2021). Their ability to penetrate cancer cells and induce cytotoxicity makes them promising candidates for targeted cancer therapies. Additionally, functionalized AgNPs conjugated with biomolecules such as flavonoids or proteins improve their specificity, reducing the risk of adverse effects in normal cells while enhancing their therapeutic efficacy.

Beyond oncology, AgNPs have been successfully integrated into antimicrobial coatings for medical implants, bandages, and wound dressings to prevent bacterial infections (González-Carter et al. 2022). Their capacity to damage bacterial cell membranes, produce reactive oxygen species (ROS), and obstruct vital biomolecules accounts for their broad-spectrum antibacterial activity.. This has led to the development of AgNP-based antimicrobial agents to combat antibiotic-resistant bacteria. Furthermore, AgNPs have been utilized in biosensing technologies for the detection of biomolecules and pathogens, aiding in early disease diagnosis and monitoring.

Despite their promising biomedical applications, AgNPs present several limitations that need to be addressed before widespread clinical use. One of the major concerns is their potential toxicity and long-term biocompatibility (Zhang et al. 2020). Studies have shown that prolonged exposure to AgNPs can lead to oxidative stress, DNA damage, and inflammation in healthy tissues, raising concerns about their safety in long-term therapeutic applications. Moreover, variations in AgNP synthesis methods can result in inconsistent physicochemical properties, affecting their biological interactions and therapeutic outcomes. To overcome these challenges, further research is required to develop standardized synthesis protocols, improve nanoparticle stability, and assess their pharmacokinetics and toxicity in clinical settings.

2.8 Toxicological and Safety Considerations of AgNPs

Concerns about the long-term safety and possible toxicity of silver nanoparticles (AgNPs) have been raised by their extensive use in biological applications. AgNPs have amazing antibacterial and anticancer qualities, but they can also have unexpected lethal effects when they interact with biological systems. Concerns regarding the biocompatibility and safe dose levels of AgNPs have been raised by studies that have demonstrated their ability to cause oxidative stress, DNA damage, and inflammatory responses in healthy cells (J. Zhang et al. 2021). AgNPs' tiny size and high reactivity enable them to pass through cell membranes and gather in essential organs, where extended exposure may cause systemic toxicity.

One of the key toxicological concerns associated with AgNPs is their ability to generate reactive oxygen species (ROS), which can disrupt cellular homeostasis and trigger apoptosis in both cancerous and healthy cells (Ebrahimzadeh et al. 2021). Additionally, AgNPs have been reported to interfere with mitochondrial function, leading to decreased ATP production and energy metabolism dysfunction. The accumulation of AgNPs in the liver, kidneys, and lungs has been observed in animal studies, suggesting the need for further investigation into their biodistribution and clearance mechanisms to mitigate long-term toxicity risks.

To address these safety concerns, researchers are exploring strategies to enhance the biocompatibility of AgNPs. Surface modifications, such as coating AgNPs with natural biomolecules like flavonoids, proteins, or polysaccharides, have been shown to reduce toxicity while preserving therapeutic efficacy (Rai et al. 2022). Moreover, standardized guidelines for AgNP synthesis, characterization, and safe dosage levels are essential to ensure their safe use in clinical applications. Ongoing research and regulatory efforts aim to establish comprehensive toxicity assessment frameworks, paving the way for the responsible and safe application of AgNPs in nanomedicine.

2.8.1 Potential risks of AgNP accumulation in biological systems

Nanoparticles have garnered significant attention for their diverse applications in biomedicine due to their unique properties, including high surface reactivity and tunable physicochemical characteristics (Ghobashy et al. 2024). Among the commonly studied nanoparticles, silver (AgNPs), gold (AuNPs), and zinc oxide nanoparticles (ZnONPs) are particularly prominent.

Silver nanoparticles (AgNPs) have garnered significant attention due to their potent anticancer properties, strong oxidative stress induction, and broad-spectrum antimicrobial activity (Fahim et al. 2024). Additionally, AgNPs synthesized through eco-friendly methods offer advantages such as cost-effectiveness and enhanced stability (Sofi et al. 2021). However, despite these benefits, excessive concentrations of AgNPs may lead to cytotoxic effects and environmental accumulation, raising concerns regarding their biosafety and potential long-term ecological impact (Nie et.al, 2023).

Gold nanoparticles (AuNPs) are widely recognized for their exceptional biocompatibility, minimal cytotoxicity, and outstanding optical properties, making them highly suitable for biomedical applications such as imaging and drug delivery (Hammami et al. 2021). However, despite these advantages, their practical use is often restricted due to their high production cost (Kumalasari et al. 2024). Additionally, AuNPs exhibit comparatively weaker antimicrobial activity than silver nanoparticles, which may limit their effectiveness in antimicrobial applications (Sa'ed et al. 2024).

Zinc Oxide nanoparticles (ZnONPs) have been widely studied for their potent antibacterial and anticancer properties, along with their excellent UV light absorption capabilities (Anjum et al. 2021). Additionally, ZnONPs are considered cost-effective and biodegradable, making them a sustainable option for various applications (J et al. 2024). However, their stability can be compromised depending on the synthesis method used, and in certain cases, ZnONPs may trigger oxidative stress in cells, raising concerns about their potential cytotoxic effects (Maheswaran et al. 2024).

Among the three types of nanoparticles, AgNPs are often regarded as the most suitable for medical applications due to their potent antimicrobial properties, cost-effectiveness, and compatibility with environmentally friendly synthesis methods. Additionally, AgNPs demonstrate significant cytotoxic effects against cancer cells, making them a versatile candidate for various therapeutic applications (Rabbi et al. 2024).

2.8.2 Strategies to enhance biocompatibility and minimize toxicity

To ensure the safe biomedical application of AgNPs synthesized using flavonoid compounds, their biocompatibility and toxicity must be carefully evaluated and optimized (Jangid et al. 2024). Various strategies can be implemented to enhance biocompatibility and minimize cytotoxic effects, with a particular focus on surface modifications, controlled synthesis parameters, and biocompatible coatings (Uneputty et al. 2021).

One effective approach involves functionalizing AgNPs with biocompatible materials, such as proteins, polysaccharides, or polyethylene glycol (PEG), which enhance stability and reduce aggregation, thereby lowering cellular toxicity (Pangli et al. 2021). Additionally, as nanoparticle size and shape significantly influence cytotoxicity, precise control over these parameters is crucial, as smaller nanoparticles generally exhibit higher surface reactivity and increased toxicity (Abbasi et al. 2023).

Optimizing synthesis conditions, including pH, temperature, and flavonoid concentration, is another key strategy to regulate nanoparticle properties, ensuring controlled release and reduced cytotoxicity (Ansari et al. 2023). Moreover, employing green synthesis approaches such as utilizing plant-derived reducing agents like quercetin, rutin, and vitexin eliminates the need for hazardous chemicals commonly used in conventional nanoparticle fabrication, further enhancing biocompatibility (Alsaiani et al. 2023).

Another critical factor in reducing toxicity is modifying the zeta potential, as maintaining a high surface charge can improve cellular uptake, inhibit aggregation, and lower cytotoxic effects (Németh et al. 2022). Collectively, these strategies including biopolymer coatings, precise control over synthesis parameters, and the use of environmentally friendly reducing agents significantly improve the biocompatibility of AgNPs, ensuring their safe and effective application in biomedical fields.

2.9 Summary of Literature Review

This chapter reviewed current knowledge on *Euphorbia milii* (mahkota duri), its phytochemical constituents, and its application in the green synthesis of silver nanoparticles (AgNPs). The literature shows that *E. milii* leaves contain a rich profile of flavonoids, tannins, phenolics, and terpenoids that can act as both reducing and stabilizing agents during nanoparticle formation. Among the identified flavonoids, rutin has been highlighted as a dominant compound with strong antioxidant and metal-chelating capacities, making it an ideal candidate for environmentally friendly nanoparticle synthesis (Kubavat et al., 2022).

Green synthesis of AgNPs using plant extracts offers a sustainable alternative to chemical and physical approaches, minimizing toxic by-products and improving biocompatibility (Iravani et al., 2019). Characterization studies commonly employ UV–Vis spectroscopy, FTIR, and SEM to confirm the formation, size, and morphology of nanoparticles. The reviewed works reported typical AgNP surface plasmon resonance (SPR) peaks between 420–440 nm, FTIR signals of –OH and C=O groups indicating capping by biomolecules, and particle sizes within 40–100 nm (Kumar et al., 2020). Previous biological assessments have shown that flavonoid-mediated AgNPs exhibit enhanced stability and reduced cytotoxicity compared with chemically synthesized AgNPs (Wu et al., 2020; Nel et al., 2006).

CHAPTER 3

METHODOLOGY

3.1 Chemical Reagent

The chemical that was used for this research are described in Table 3.1.

Table 3.1 List of Chemicals

Chemicals	Molecular Formula	Molecular (g/mole)	Purify (%)	Manufacture
Hexane	C ₆ H ₁₄	86.18	95	R&M Chemical
Chloroform	CHCl ₃	119.37	99	R&M Chemical
Ethyl acetate	C ₄ H ₈ O ₂ (CH ₃ COOCH ₂ CH ₃)	88.11	99	R&M Chemical
Acetone	(CH ₃) ₂ CO	58.08	99.9	R&M Chemical
Methanol	CH ₃ OH	32.04	99.8	R&M Chemical
Silver nitrate	AgNO ₃	169.87	99.8	R&M Chemical
Dimethyl sulfoxide	C ₂ H ₆ OS	78.13	99.5	R&M Chemical
MTT (3-(4,5-Dimethyl-	C ₁₈ H ₁₆ BrN ₅ S	414.32	98	R&M Chemical

2-
thiazolyl)-
2,5-
diphenyl-
tetrazolium
bromide)

3.2 Apparatus

The apparatus that were used for this research were beaker (50 mL, 100 mL, 250 mL, 500 mL and 1L), conical flask (50 mL, 100 mL and 500 mL), micropipette, micropipette tips (yellow and blue), glass rod, test tubes, dropper, filter paper, filter funnel, round bottom flask 250 mL, test tube rack, vials 20 mL, syringe 5 mL, syringe filter 0.22µm, volumetric flask 50 mL, cuvette, spatula, ITO plate, cell culture flask (small and medium), serological pipette (2 mL, 5 mL, and 10 mL), cryogenic vial, centrifuge tube (15 mL and 50 mL), reagent bottle (500 mL and 1 L), 96-well plate, 24-well plate, cell counter slide and cell scraper.

3.3 Plant Material

The fresh plant sample of *Euphorbia milii* was collected from Kota Sarang Semut, Kedah, Malaysia (5.7031° N, 100.4978° E) in November 2020. The collected specimen was authenticated by a senior laboratory assistant from the Biological Laboratory, Faculty of Science and Marine Environment, Universiti Malaysia Terengganu.

3.4 Sample Preparation and Extraction

A 70 g sample was first rinsed thoroughly under running tap water and left to air dry at room temperature for seven days. Once dried, it was ground into a fine powder using a grinder. The powdered plant material was then soaked in six different solvents with varying polarities (hexane, chloroform, ethyl acetate, acetone, methanol, and distilled water) from Sigma-Aldrich, US, at room temperature for 72 hours. After soaking, each extract was concentrated using a Büchi Rotavap R-200 rotary evaporator (Switzerland) under reduced pressure at 35–40 °C for 15–30 minutes. The resulting

six crude extracts were weighed and stored in a chiller at 4 °C for further analysis. Figure 3.1 provides a flowchart of the sample preparation and extraction process.

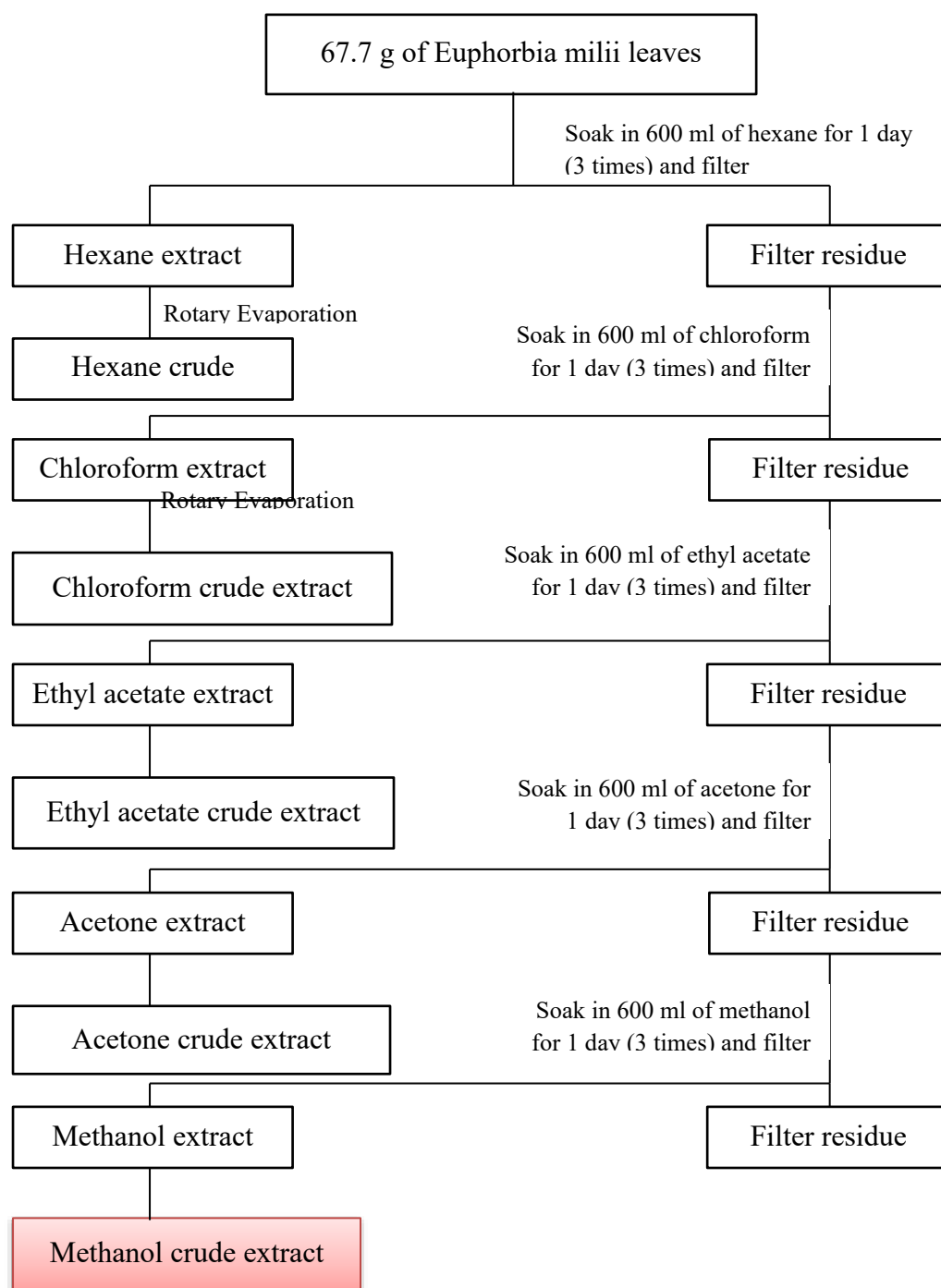


Figure 3.1 Flowchart of sample preparation and extraction process

3.5 Total Flavonoid Content (TFC)

Aluminium chloride colorimetric technique was used in the determination of TFC. About 20 μL of samples (concentration 5 mg/mL) were placed in a 96-well microplate and 120 μL of distilled water was added to each sample extract, followed by 10 μL of 5% sodium nitrite and incubated for 5 min. Then, 10 μL of 10% aluminium chloride was added to each sample extract. After 6 min of incubation, 40 μL of 1 M sodium hydroxide was added and incubated for another 15 min. The absorbance was then measured at 520 nm. The analysis was conducted for all samples. The standard curve was prepared by using quercetin standard. The results were expressed as mg quercetin equivalent (QE)/g extract (Sari et al. 2023).

3.6 Nuclear Magnetic Resonance (NMR)

The structural configuration of the extracts obtained were elucidated using Nuclear Magnetic Resonance (NMR). NMR spectra (1D and 2D) were recorded on Bruker 400 and DMX 600 NMR spectrometer (Makmal Penyelidikan, Institut Marin Bioteknologi, Universiti Malaysia Terengganu). The solubility for deuterated solvent of each extract were confirmed before the analysis. Most of the extracts obtained soluble in deuterated methanol (CD_3OD). The raw data obtained from the analysis were interpreted with Bruker Top Spin 4.0 software. 1D NMR was used to determine the number of protons (^1H) and thirteen-carbon (^{13}C) in the molecule. 2D NMR was used to provide information about the correlation of proton to proton (COSY), proton to carbon (HMQC) and the long-range protons-carbons connectivity (HMBC).

3.7 Chemical Profiling of *Euphorbia milii* Leaves Crude Extract

The chemical profiling of *E. milii* leaf crude extract is essential for identifying its bioactive constituents and understanding their potential pharmacological and nanotechnological applications. This process involves the comprehensive analysis of secondary metabolites, such as flavonoids, phenolics, alkaloids, tannins, terpenoids, and saponins, which contribute to the plant's antioxidant, antimicrobial, and medicinal properties. To achieve accurate chemical profiling, various analytical techniques,

including nuclear magnetic resonance (NMR) spectroscopy, total flavonoid content (TFC), high-performance liquid chromatography (HPLC), and Fourier transform infrared spectroscopy (FTIR), are employed. These techniques help in identifying the major phytoconstituents responsible for the biological activities and nanoparticle synthesis potential of *E. milii*. Understanding the chemical composition of the crude extract provides valuable insights into its role as a natural reducing and stabilizing agent in green nanoparticle synthesis, as well as its applications in biomedical and therapeutic research.

3.7.1 High Pressure Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a widely used analytical technique for identifying, quantifying, and separating bioactive compounds in plant extracts (Kumar 2017). In this study, HPLC analysis is employed to determine the flavonoid composition of *E. milii* leaf crude. The method involves the preparation of the sample and solvent, followed by the preparation of standard solutions for accurate compound identification and quantification.

Sample and Solvent Preparation

For HPLC analysis, 2 mg of crude extract from different solvents (ethyl acetate, acetone, and methanol) was dissolved in 1 mL of HPLC-grade methanol and subsequently filtered through a 0.22 µm membrane filter to eliminate any particulate matter. This step ensures a clear, impurity-free solution, making it suitable for injection into the HPLC system. For solvent preparation, 1% formic acid was added to both acetonitrile (ACN) and Milli-Q water to optimize the chromatographic separation.

Standard Preparation

For compound identification and quantification, standard solutions of flavonoids, including quercetin, rutin, vitexin, apigenin, myricetin and kaempferol, were prepared at 2 mg/ml concentrations using HPLC-grade solvents. Each standard compound was dissolved in methanol and diluted to obtain a calibration curve for accurate quantification. The prepared standard solutions were then filtered through a 0.22 μm membrane filter before analysis. The retention times and peak areas of the standard compounds serve as references for identifying corresponding peaks in the *E. milii* extract chromatogram.

3.8 Green Synthesis of Silver Nanoparticles (AgNPs)

Building on the method modified by Vishwanath and Negi (2021), silver nanoparticles (AgNPs) were synthesized using *E. milii* leaf extract as the reducing agent and silver nitrate (AgNO_3) as the precursor. To begin, 10 mg of the crude extract was dissolved in 10 ml of Milli-Q water and then filtered using a 0.22 μm syringe filter. Next, 5 ml of this filtered solution was mixed with 50 ml of a 1 mM AgNO_3 solution. The mixture was left undisturbed in the dark at room temperature for 24 hours, during which the color changed from colorless to brown, indicating nanoparticle formation. Figure 3.2 provides a visual overview of the entire process.

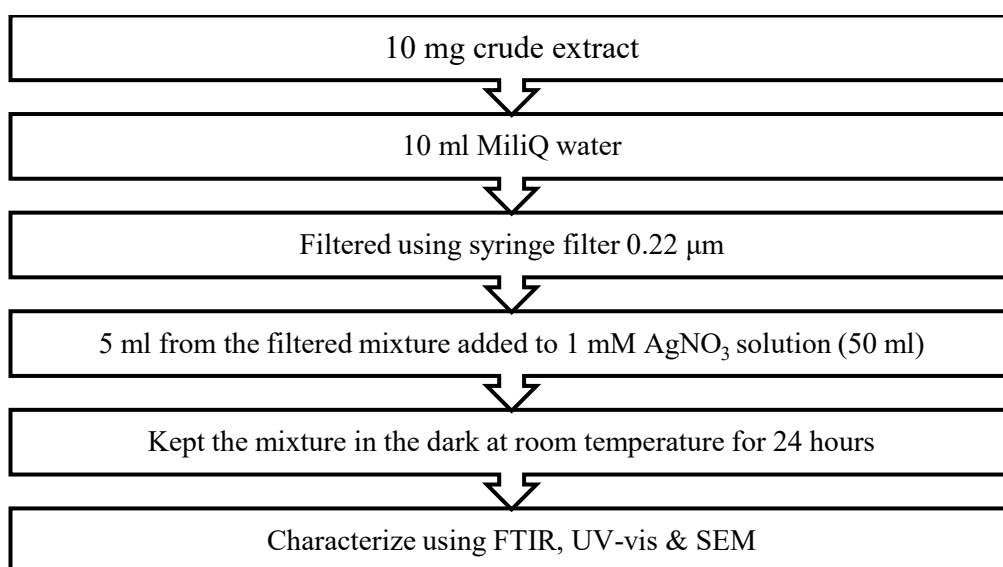


Figure 3.2 Flow chart for green synthesis route AgNPs

3.8.1 Characterization of Silver Nanoparticles (AgNPs)

The characterization of AgNPs is a crucial step in evaluating their physicochemical properties, stability, and suitability for biomedical applications. Proper characterization ensures a comprehensive understanding of the size, shape, surface morphology, and functional groups involved in the synthesis process (Lin et al. 2014). To achieve this, various analytical techniques are employed, including Fourier Transform Infrared Spectroscopy (FTIR) for functional group identification, UV-Visible Spectroscopy (UV-Vis) for optical properties, and Scanning Electron Microscopy (SEM) for morphological analysis. These techniques collectively provide insights into the formation, stability, and potential applications of AgNPs synthesized using *E. milii* leaf extract and flavonoids compound.

3.8.1.1 Fourier Transform Infrared Spectroscopy (FTIR)

In this study, Fourier Transform Infrared Spectroscopy (FTIR) was used to analyse the functional groups of various materials, including organic, polymeric, and some inorganic substances. This technique works by using infrared light to scan the samples, helping to identify their chemical properties. By producing spectra that generate a unique molecular fingerprint, FTIR can effectively screen and analyse samples for various components. It is a valuable instrument for detecting functional groups and characterizing covalent bonding information. In this work, FTIR analysis was performed using a Perkin Elmer Spectrum 100 with the ATR method. Before starting the ATR-FTIR spectroscopy, the ATR crystal was thoroughly cleaned, and the infrared background spectrum was recorded. Then, a sample (1 mg/mL in acetone) was placed on the crystal, and the measurement process began. All crude extracts and AgNPs products were performed for all analysed samples. All spectra obtained were converted to ACSII files prior to the multivariate data analysis.

3.8.1.2 Ultraviolet-Visible Spectroscopy (UV-Vis)

UV-Visible spectroscopy is an absorption spectroscopy where the atoms and molecules undergo electronic transitions. It has a wide range of applications including quantification during the aggregation process of conjugated polymer solution. The absorption of photons was calculated in transitions from ground state to excited state. Electronic transitions via the Born-Oppenheimer Approximation of stationary or slow-moving nuclei and rapidly moving electrons follow the Franck-Condon principle. Absorbance of UV-visible spectroscopy generally follows the Beer's Law, which states that absorbance is directly proportional to its concentration. The concentration measurements can be made at the wavelengths where there is the largest change in absorbance per unit (Zhao et al. 2019).

3.8.1.3 Scanning Electron Microscope (SEM)

The scanning electron microscope (JEOL 6360 LA) was used to morphology of observe the AgNPs. The sample was prepared by putting the solution of AgNPs on the surface of ITO plate. Then, it was sonicated for 15 min and keep it dried before followed by the application of a gold metal coating to the sample. The particle size of the AgNPs was examined at 10 000 X and 15,000 X, with an acceleration rate of 15 kV at a scale of 15 mm (Fischer et al. 2012).

3.9 Cell Culture

Cell culture techniques are essential for evaluating the biological effects and cytotoxicity of silver nanoparticles (AgNPs) synthesized using flavonoid. In this study, Vero cells, a widely used mammalian cell line, are employed to assess the cytotoxic potential of AgNPs through in vitro assays. Proper preparation of cell culture media, reagents, and subculturing techniques is crucial to ensure optimal cell growth, viability, and reproducibility of experimental results.

3.9.1 Media and Reagents Preparation

The preparation of cell culture media and reagents is a fundamental step in maintaining healthy cell growth. Different media formulations, including 10% Dulbecco's Modified Eagle Medium (DMEM) are prepared to provide essential nutrients and maintain physiological conditions for Vero cell culture. Additionally, MTT reagent, a key component for cytotoxicity assays, is prepared to evaluate the metabolic activity of cells upon exposure to AgNPs.

Preparation of 10% DMEM:

To prepare 10% DMEM, Dulbecco's Modified Eagle Medium (DMEM) is enriched with 10% fetal bovine serum (FBS) to support cell growth, along with 1% penicillin-streptomycin (PenStrep) and 1% non-essential amino acids (NEAA) to maintain sterility and optimize cell health. The mixture is carefully combined under sterile conditions and passed through a 0.22 μm membrane filter to remove any contaminants. Once prepared, the 10% DMEM is divided into sterile bottles and stored at 4°C to keep it stable and ready for use in cell culture experiments.

MTT Reagent:

The MTT reagent is prepared by dissolving 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) per milliliter of phosphate-buffered saline (PBS) to create a 5 mg/mL stock solution. The solution is then filtered using a 0.22 μm syringe filter to ensure sterility and remove any undissolved particles. Once filtered, the reagent is aliquoted into light-protected tubes or amber vials to prevent light-induced degradation. For storage, the MTT solution is kept at -20°C for long-term use or 4°C for short-term applications. Before use, the reagent is brought to room temperature and protected from direct light to maintain its stability and effectiveness for cytotoxicity assays.

3.9.2 Vero Cell Line Propagation and Subculture

The Vero cell line, originating from the kidney epithelial cells of the African green monkey (*Cercopithecus aethiops*), is commonly used in biomedical research and cytotoxicity tests because of its strong growth and adaptability in lab conditions. To ensure the cells remain healthy and viable for experiments, proper cultivation and subculture techniques are crucial.

Cell Thawing and Initial Seeding

To revive frozen Vero cells, they are quickly thawed in a 37°C water bath and then transferred into a sterile centrifuge tube containing pre-warmed complete growth medium (10% DMEM with 10% FBS and 1% PenStrep). The mixture is then centrifuged at 200 x g for 5 minutes to separate the cells, which are then resuspended in fresh medium. After that, the cells are seeded into a T-25 culture flask and incubated at 37°C in a humidified environment with 5% CO₂, allowing them to attach and grow.

Cell Growth Monitoring and Maintenance

Cells are observed daily under an inverted microscope to monitor their morphology and confluency. The culture medium is replaced every 48 hours to maintain optimal nutrient availability and remove metabolic waste. Once the cells reach 70–80% confluency, subculturing is performed to prevent overgrowth and maintain cell viability.

Subculturing Process

For subculture, the spent medium is removed, and the cell monolayer is gently washed with sterile phosphate-buffered saline (PBS) to remove residual serum proteins. 1 ml TrypLE is then added to detach the adherent cells, and the flask is incubated at 37°C for 2 minutes until cells begin to lift. The enzymatic reaction is neutralized by adding fresh complete medium, and the cell suspension is centrifuged at 500 x g for 3 minutes. The cell pellet is resuspended in fresh medium, and the cells are reseeded into new culture flasks at the appropriate dilution.

Storage and Cryopreservation

To preserve the Vero cell line for future use, cells are harvested at 80–90% confluency, resuspended in freezing medium (10% DMSO in FBS), and slowly frozen at -80°C before being transferred to liquid nitrogen for long-term storage. This ensures that cells remain viable and retain their biological properties when revived for subsequent experiments.

3.9.3 Viable Cells Count by Trypan Blue

The culture media of Vero cells with over 90% confluency in a small flask was removed. Subsequently, the cells were washed with 3 ml of PBS and the PBS was removed. Then, 1 ml of TrypLE was added to the flask and gently shaken, followed by 1 minutes and 30-second incubation of the cells at a 37°C incubator. The cells were observed under a microscope to confirm detachment. To halt trypsinization, 3 ml of fresh DMEM medium was added, and the cells were re-suspended before being transferred into a 15 ml centrifuge tube. Centrifugation was carried out at 500 x g for 3 minutes, and the supernatant was removed. The cell pellet was gently re-suspended in 1 ml of DMEM, and 20 µl of this suspension was aliquoted into an Eppendorf tube. Subsequently, 20 µl of trypan blue was added to the tube containing the cells. Then, 10 µl of the cell suspension was pipetted onto a haemocytometer for examination under a microscope. Figure 3.3 shows the flow chart of the overall process of cell count. Clear cells on the grid were counted (Figure 3.4), and the cell concentration was calculated using a formula:

$$\text{Concentration of cells} = \text{Average of viable cell per square} \times \text{dilution factor} \times 10^4 \quad (3.1)$$

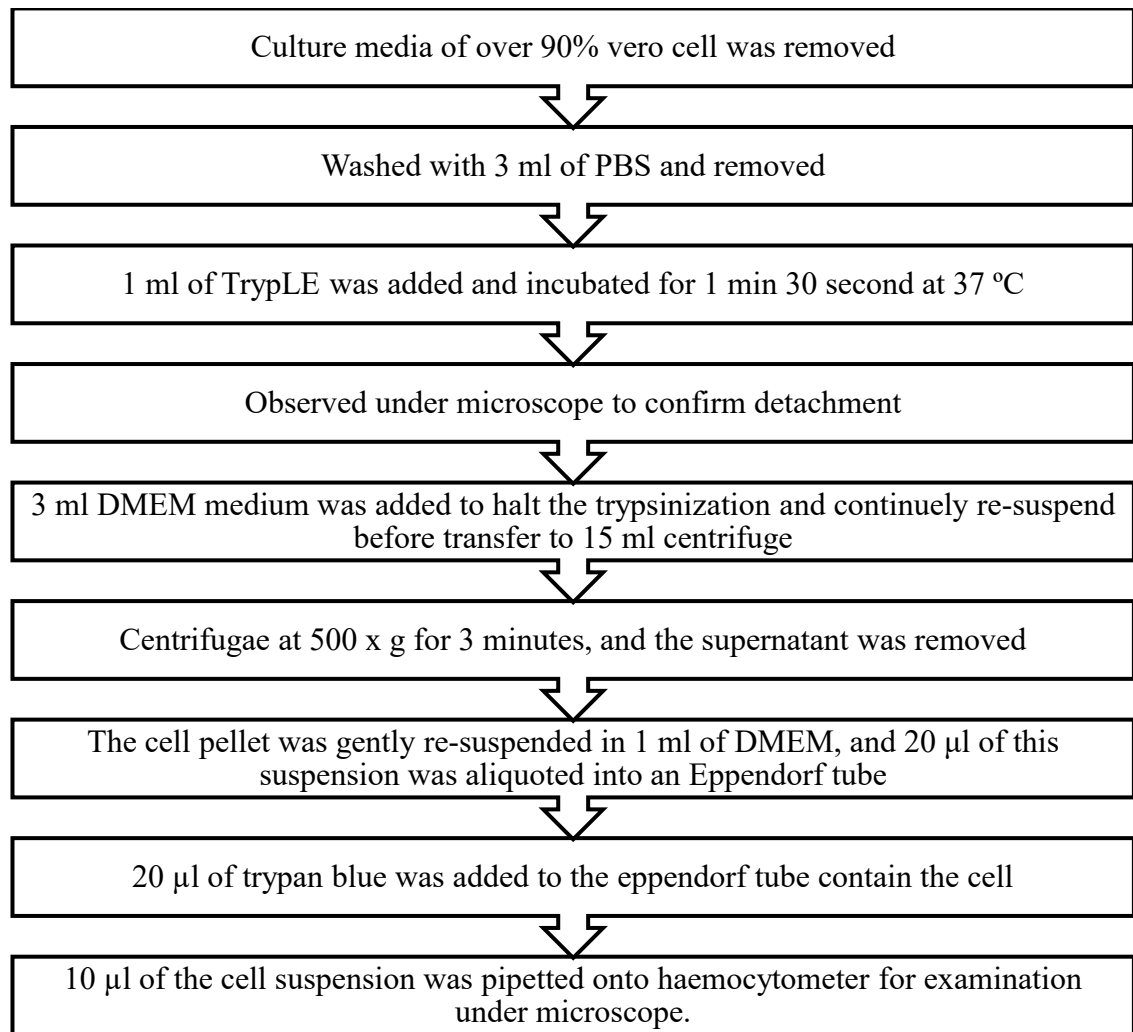


Figure 3.3 Flow chart for cell count

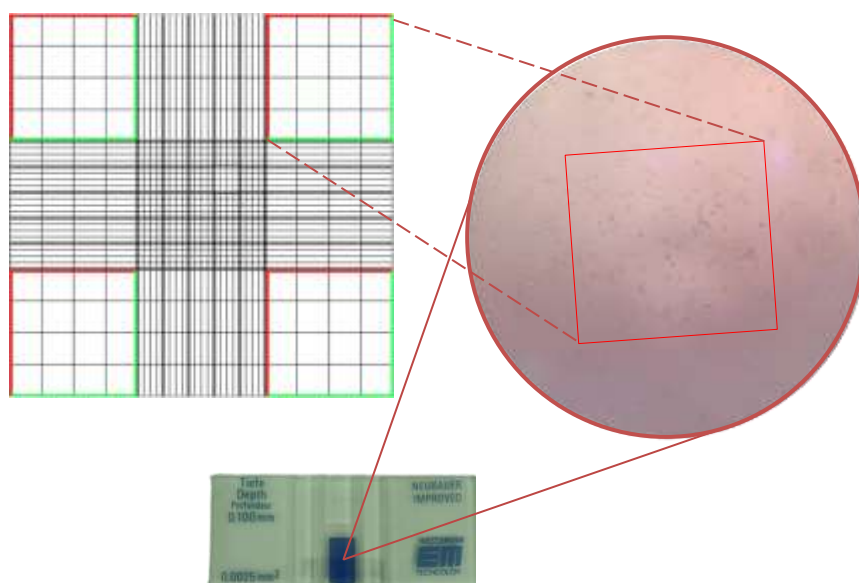


Figure 3.4 Cell in haemocytometer

3.10 Cytotoxicity MTT Assay

To initiate the cell seeding process, the cells were diluted with DMEM medium to achieve a concentration of 1×10^4 cells/well for Plate 1 (analysed at 24 hours) and Plate 2 (analysed at 48 hours), and 1×10^3 cells/well for Plate 3 (analysed at 72 hours), with the volume adjusted as needed. After thorough mixing, 100 μ l of the cell suspension was pipetted into each well of a flat-bottom 96-well microtiter plate. Subsequently, the cells were incubated overnight at 37°C in a 5% CO₂ environment.

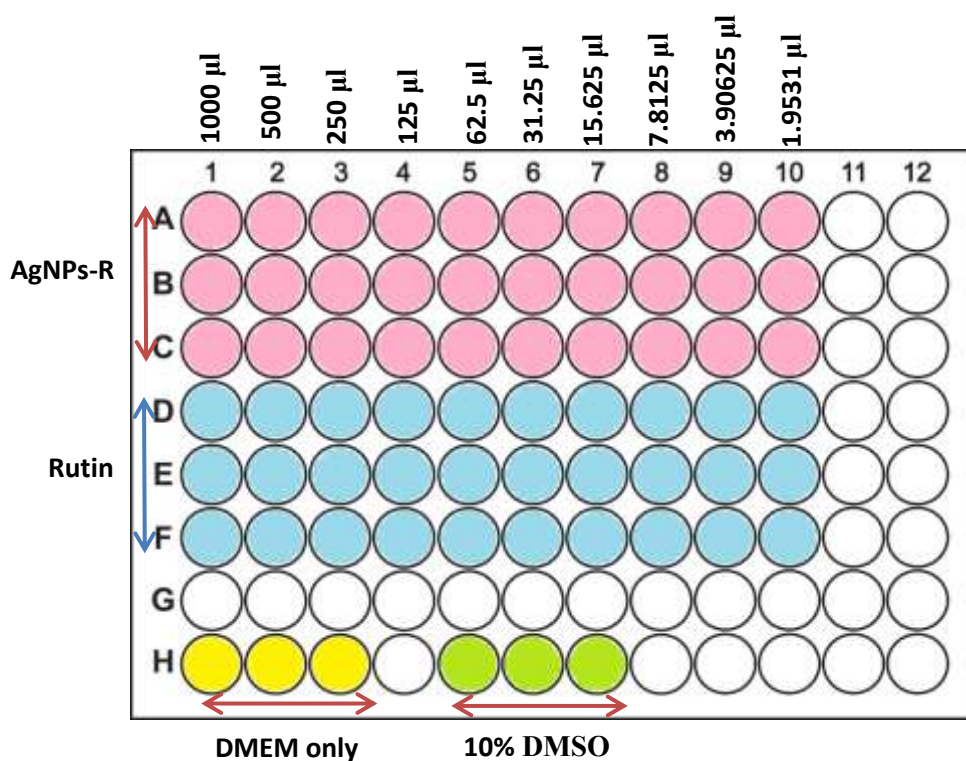


Figure 3.5 Layout of 96-well plate of cytotoxicity treatment

On the second day, various concentrations of the sample were prepared by diluting AgNPs-rutin and rutin in DMEM medium (1000 – 1.9531 μ g/ml), with proper labelling of all tubes. A negative control was established by treating cells with DMEM medium alone, while a positive control employed 10% DMSO (50 μ l DMSO in 450 μ l DMEM). Before conducting the experiment, the cells' confluency (80-90%) and morphology were carefully verified. Plates were labelled according to the layout of the 96-well treatment as shown in Figure 3.5. To prevent cell drying, the culture medium

was removed from every 3 wells. Then, 100 μ l of the sample dilution was pipetted into each well, with triplicate wells for each concentration. The plates were incubated for 24, 48, and 72 hours at 37°C in a 5% CO₂ environment.

On the third day, the test solution was removed, and the wells were washed twice with 100 μ l of fresh DMEM for 2-3 minutes. Following this, 100 μ l of fresh DMEM was added to each well before proceeding with the MTT assay. 10 μ l of MTT solution were added to each well and mixed thoroughly. The plates were then incubated for 4 hours at 37°C in the dark, covered with aluminium foil. After the incubation period, all media were removed, and 100 μ l of DMSO was added to each well, followed by a 10-minute incubation at room temperature covered by aluminium foil. The plates were then placed on a plate shaker for 5 minutes at 300 rpm, and the absorbance was measured at 570 nm using microplate reader. The MTT treatment was conducted for samples incubated for 48 and 72 hours the next day. The sample availability was limited, allowing the assay to be conducted only up to the MTT method (without IC₅₀ determination).

Table 3.2 Experimental Design and Replicates for Cytotoxicity (MTT) Assay of *Euphorbia milii*-Derived AgNPs and Rutin

Sample Code	Treatment Type	Concentration Range (μ g/mL)	Dilution Method	Exposure Time (h)	No. of Replicates per Concentration
C+ (Positive Control)	10% DMSO	-	-	24,48,72	3
C- (Negative Control)	Untreated cells (medium only)	-	-	24,48,72	3
Sample 1	AgNPs-Rutin	1000-1.9531	2-fold serial dilution	24,48,72	3
Sample 2	Rutin	1000-1.9531	2-fold serial dilution	24,48,72	3

Table 3.2 presents the experimental design and number of replicates used in the cytotoxicity (MTT) assay for *Euphorbia milii*-derived silver nanoparticles (AgNPs-Rutin) and pure Rutin. Two samples were tested across ten concentrations ranging from 1000 μ g/mL to 1.95 μ g/mL, prepared through a two-fold serial dilution method. The cytotoxicity was evaluated at three different exposure durations (24 h, 48

h, and 72 h). Each concentration was tested in triplicate ($n = 3$) using a 96-well plate format, and the entire experiment was independently repeated three times to ensure reproducibility and statistical reliability. The 10% DMSO solution and untreated culture medium served as positive and negative controls, respectively.

All MTT assay data were expressed as mean $\pm$ standard deviation (SD) from three independent experiments, each performed in triplicate. Statistical analysis was conducted using Microsoft Excel (Microsoft Office 365) with the Data Analysis ToolPak add-in. A one-way analysis of variance (ANOVA) was applied to evaluate differences in cell viability among various treatment concentrations and between the Rutin and AgNPs–Rutin groups.

When the ANOVA results indicated significant differences ($p < 0.05$), Tukey's post hoc test was performed using an online statistical calculator (SocSciStatistics.com) to identify which group means differed significantly. A p value less than 0.05 ($p < 0.05$) was considered statistically significant, while $p > 0.05$ indicated no significant difference. Error bars in all figures represent standard deviation (SD), which reflects the variability among replicates and the reproducibility of the experimental results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Metabolite Profiling

Metabolic profiling involves analyzing the small molecules and their intermediates in biological systems, from single cells to complex organisms, to understand how they respond to genetic changes. In this study, the metabolite profiling of *E. milii* leaf extract was carried out to identify its bioactive compounds, which are key to its medicinal properties and potential use in green nanoparticle synthesis. Various analytical techniques, including nuclear magnetic resonance (NMR), total flavonoid content (TFC), high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR), were employed to detect and characterize which extract contained most flavonoid compound. The analysis revealed the most presence of flavonoids was at three solvents extract which is ethyl acetate, acetone and methanol extract.

4.1.1 Nuclear Magnetic Resonance (NMR)

To examine and compare the flavonoid composition in six different crude extracts of *E. milii* leaves, Nuclear Magnetic Resonance (NMR) spectroscopy was utilized. This method offers in-depth insights into the chemical structure, functional groups, and molecular makeup of the extracts, making it possible to identify and measure flavonoid compounds. The study specifically analyzed ^1H NMR and ^{13}C NMR spectra, which are widely used to detect distinct proton and carbon shifts characteristic of flavonoids.

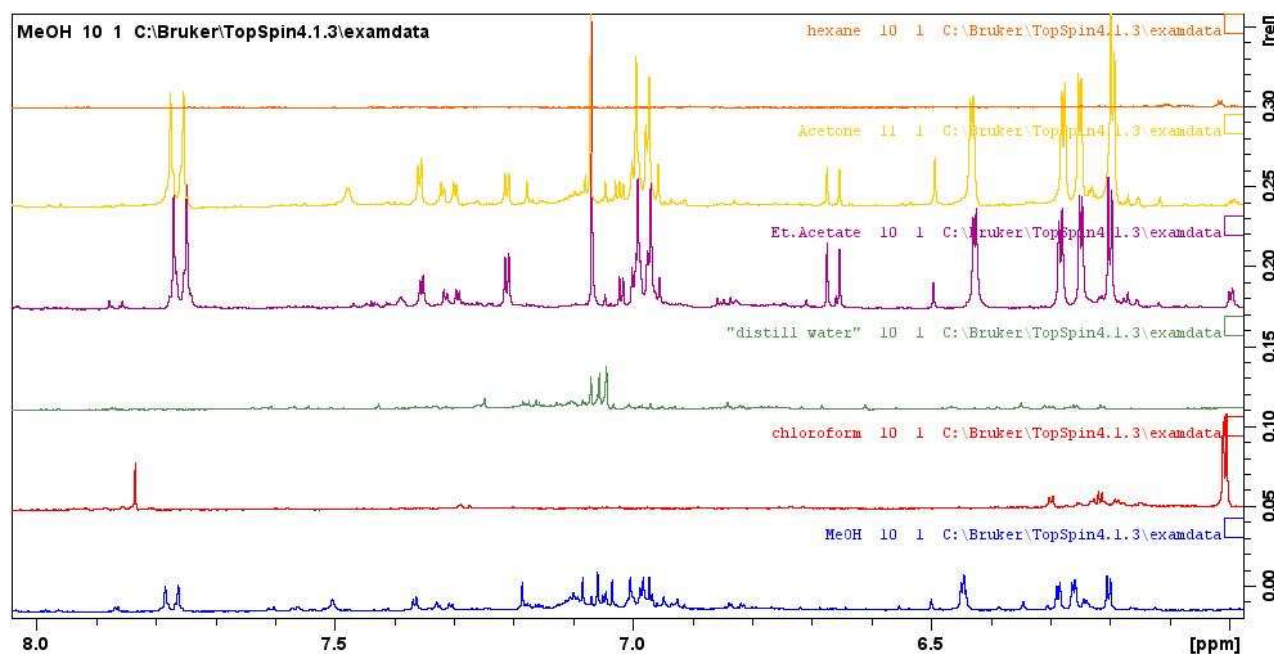


Figure 4.1 NMR analysis of crude extracts

Figure 4.1 shows the NMR analysis of six different crude extract. The ^1H NMR spectra of the six crude extracts revealed distinct chemical shift patterns in the range of 6.0–8.5 ppm, corresponding to the aromatic protons of flavonoid compounds. Among the tested extracts, only acetone, ethyl acetate, and methanol exhibited strong peak intensities within this range, suggesting a higher concentration of aromatic compounds, potentially flavonoids.

4.1.2 Total Flavonoid Content (TFC)

The total flavonoid content (TFC) analysis was subsequently performed to quantify the flavonoid concentration across six different crude extracts. As presented in Table 4.1, ethyl acetate, acetone, and methanol exhibited the highest TFC values, measuring 1180.33 ± 55.39 , 960.33 ± 27.11 , and 955.89 ± 34.96 mg QE/g, respectively.

Table 4.1 The TFC value of six differences crude extract	
Plant Extracts	TFC (mg QE/g)
Hexane	165.89 ± 32.61
Chloroform	653.67 ± 31.82
Ethylacetate	1180.33 ± 55.39
Acetone	960.33 ± 27.11
Methanol	955.89 ± 34.96
Distill water	200.89 ± 43.21

The total flavonoid content (TFC) of *Euphorbia milii* leaf extracts varied with solvent polarity, where the methanolic extract exhibited the highest flavonoid concentration (92.64 ± 1.35 mg QE g $^{-1}$), followed by acetone and ethyl acetate extracts. This finding indicates that methanol, being a highly polar solvent, effectively solubilized both polar and moderately polar flavonoids, resulting in greater extraction efficiency. Flavonoids are predominantly glycosylated and thus more soluble in polar solvents such as methanol and ethanol (Abu Bakar et al., 2017). Similar patterns were reported in other *Euphorbia* species, where methanol extracted the greatest flavonoid

content compared to less polar solvents (Sharma et al., 2020). Figure 4.2 and 4.3 shows the results with error bars (repeated 3 times) and the standard graft for quercetin as positive control.

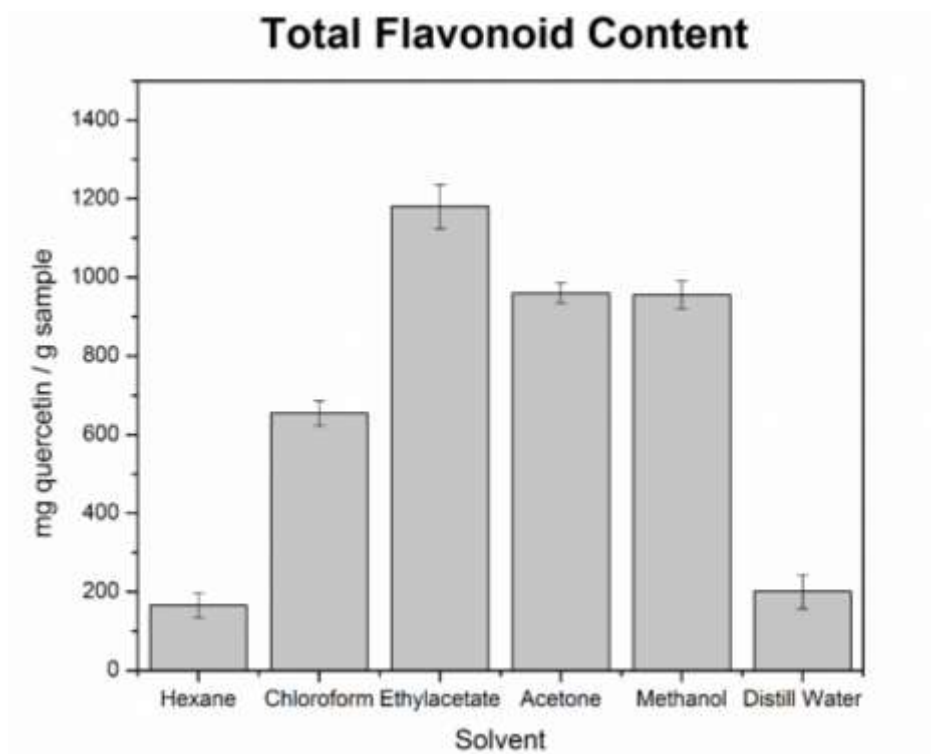


Figure 4.2 Result of total flavonoid content for 6 crude extracts

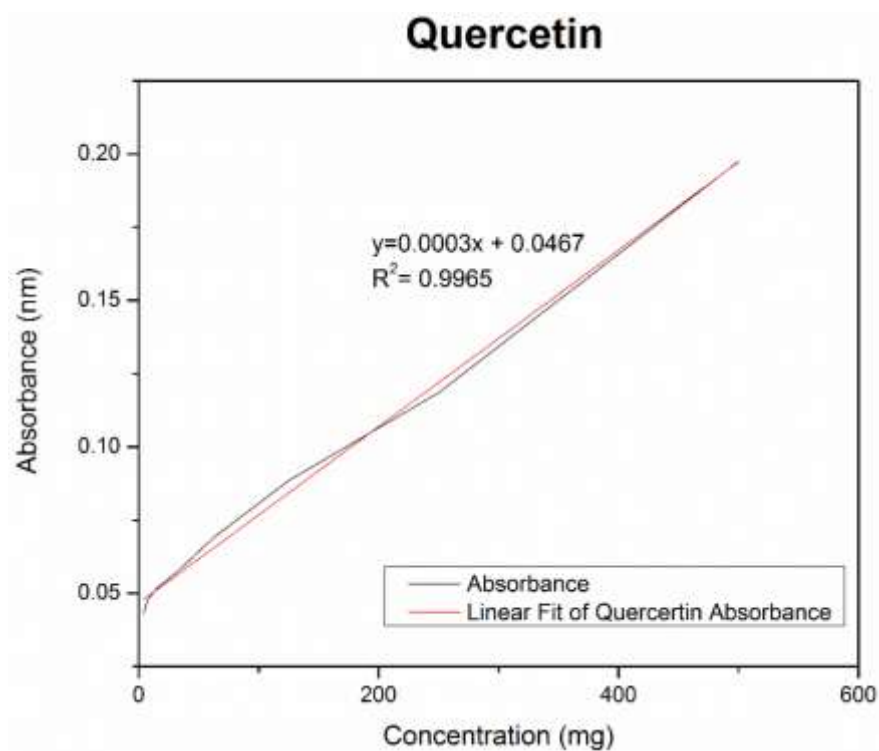


Figure 4.3 Quercetin as positive control (standard)

4.1.3 High Pressure Liquid Chromatography (HPLC)

The TFC results indicated that ethyl acetate, acetone, and methanol extracts contained the highest flavonoid content. Consequently, these three extracts were subjected to High-Performance Liquid Chromatography (HPLC) analysis to identify and confirm the presence of specific flavonoid compounds. HPLC is an advanced analytical technique that enables the precise separation, detection, and quantification of flavonoids by measuring their retention time and peak characteristics, allowing for a more comprehensive chemical profile of the extracts.

For HPLC analysis, six pure flavonoid compounds—rutin, vitexin, myricetin, quercetin, apigenin, and kaempferol—were used as standards. These reference compounds were compared against the methanol, acetone, and ethyl acetate crude extracts to determine their presence. Figures 4.4, 4.5, and 4.6 display the chromatographic profiles, illustrating the retention time and peak intensity of each crude extract in relation to the flavonoid standards. The results suggest that the methanol extract likely contains rutin, vitexin, myricetin, and quercetin, as indicated by corresponding peaks. In contrast, the acetone and ethyl acetate extracts showed lower peak intensities, suggesting a lower flavonoid concentration or limited presence of the tested compounds. Table below shows the retention time (R_t) in minutes for compounds detected in extracts with their corresponding standards.

Table 4.2 Retention time (R_t) in minutes for compounds detected in extract with their corresponding standards.

Standard compound	R_t (min) Standard	R_t (min) Methanol extract	R_t (min) Acetone extract	R_t (min) Ethyl acetate extract	References
Rutin (a)	22.347	22.326	22.355	22.480	De Lima et al. (2021)
Vitexin (b)	22.907	22.826	-	-	Ekeke & Ndukwu (2014)
Myricetin (c)	25.199	25.170	25.202	25.178	Ekeke & Ndukwu

					(2014); De Lima et al. (2021)
Quercetin (d)	26.806	26.807	-	-	Ekeke & Ndukwu (2014); De Lima et al. (2021)
Apigenin (e)	27.937	-	-	-	Hassan et al. (2023)
Kaempferol (f)	28.390	28.472	-	-	-

Previous studies have demonstrated the presence of various flavonoid compounds within *Euphorbia* species. Ekeke and Ndukwu (2014) reported the identification of quercetin in the ethyl acetate fraction of the aerial parts of *Euphorbia milii*, together with myricetin and vitexin in the whole plant extract. Similarly, Hassan et al. (2023) detected apigenin in the aerial parts of *E. milii*, further confirming the abundance of diverse flavonoids in this species. Moreover, De Lima et al. (2021) identified rutin, myricetin, and quercetin in *E. tirucalli* using HPLC–MS analysis, suggesting that these compounds are likely common across related *Euphorbia* species.

In the present study, the HPLC chromatogram of *E. milii* leaf methanol extract also exhibited peaks corresponding to rutin, myricetin, quercetin, kaempferol, and vitexin, which corroborates previous findings. Among these, vitexin, rutin, and quercetin were selected for further study due to their strong reducing and stabilizing properties. Flavonoids are known for their multiple hydroxyl (–OH) groups, which enable them to donate electrons and reduce Ag^+ ions during nanoparticle synthesis. Specifically, these three compounds possess multiple hydroxyl, ketone, and ether functional groups, along with planar aromatic structures, making them highly effective as natural reducing and capping agents. Furthermore, their well-established antioxidant and biological activities, combined with their structural diversity, provide a strong foundation for investigating their individual roles in the formation and stabilization of silver nanoparticles.

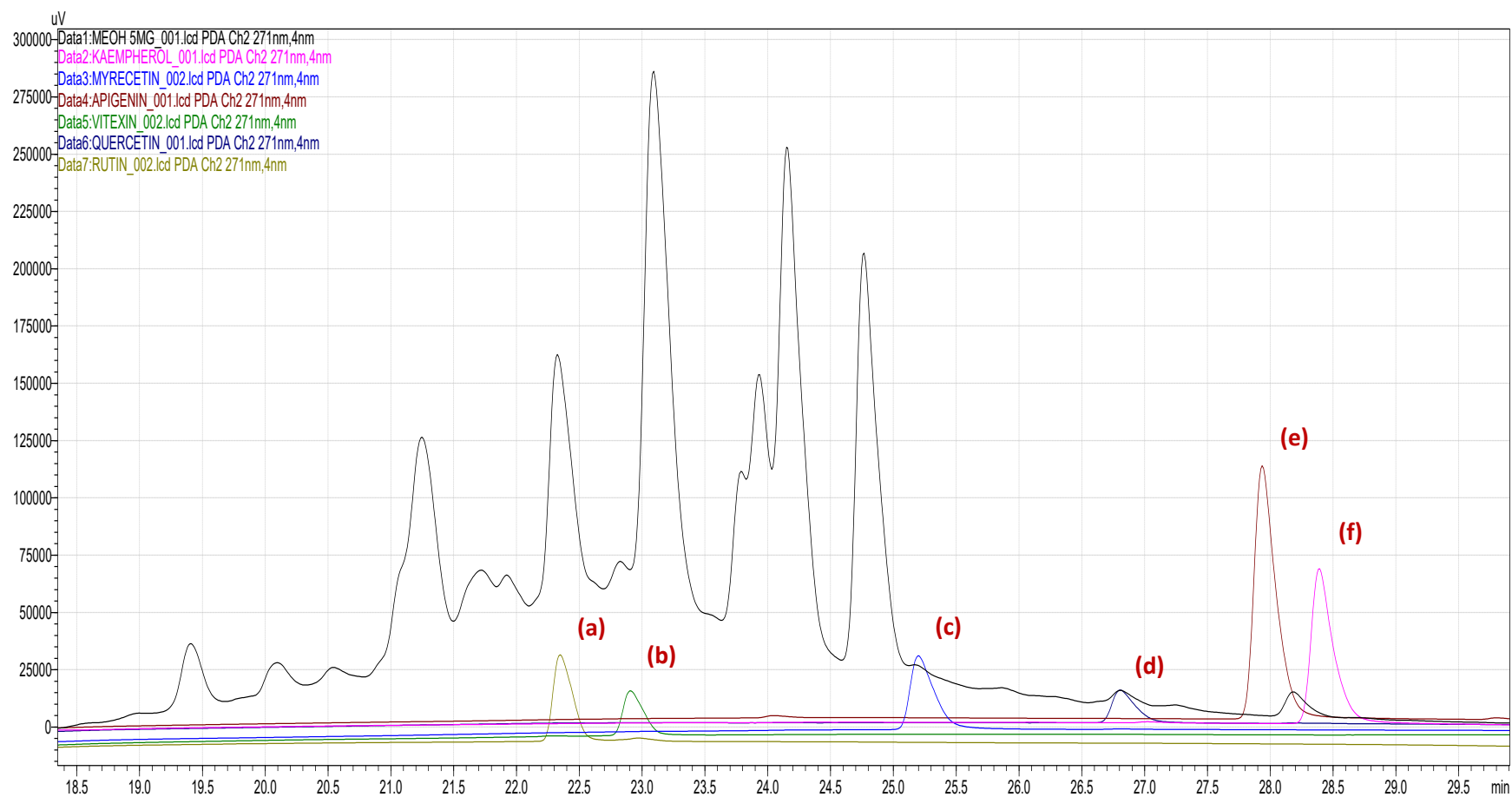


Figure 4.4 HPLC chromatogram of methanol extract with six compounds a) rutin, b) vitexin, c) myrecetin, d) quercetin, e) apigenin and f) kaempferol

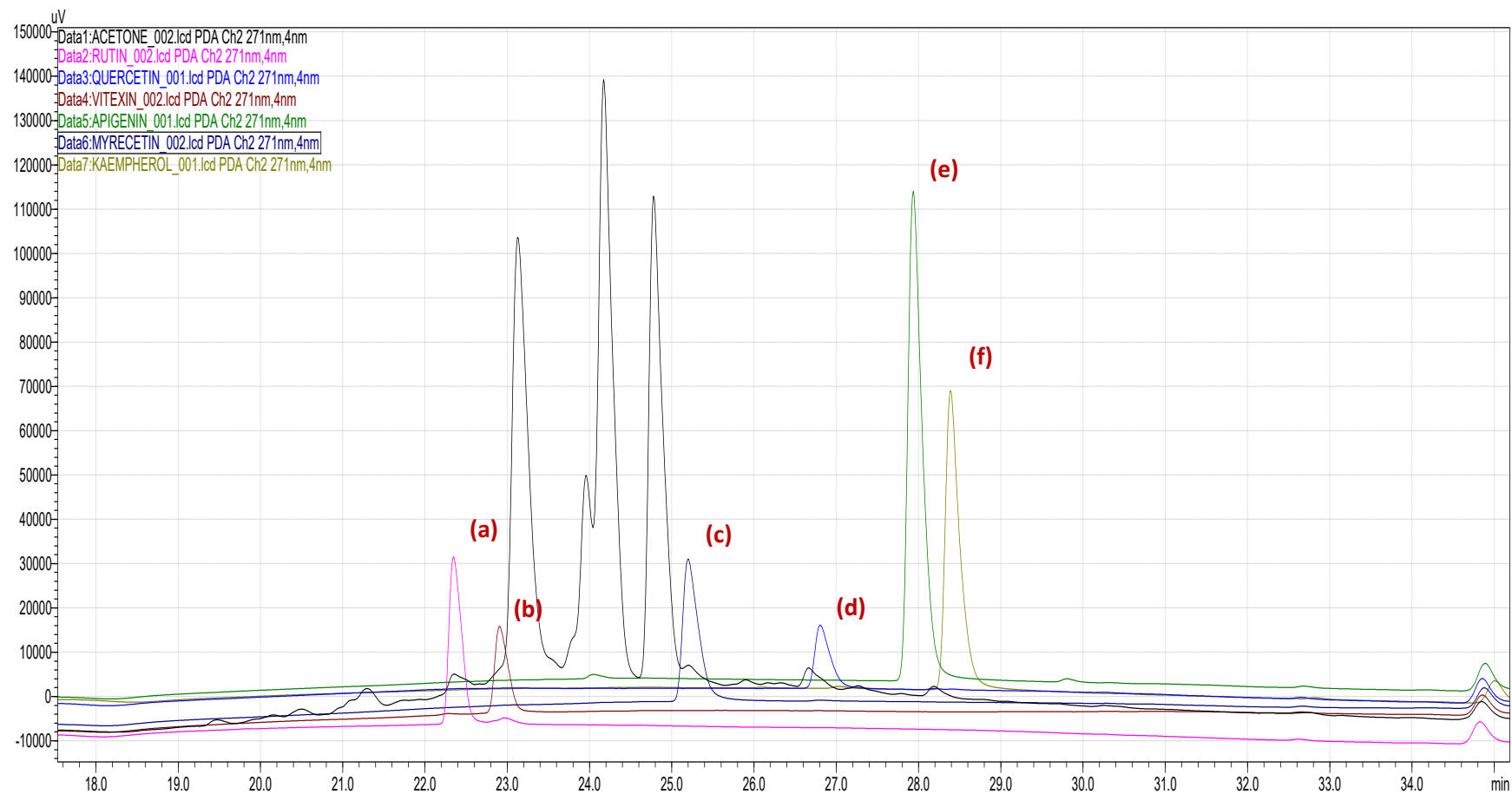


Figure 4.5 HPLC chromatogram of acetone extract with six compounds a) rutin, b) vitexin, c) myrecetin, d) quercetin, e) apigenin and f) kaempferol

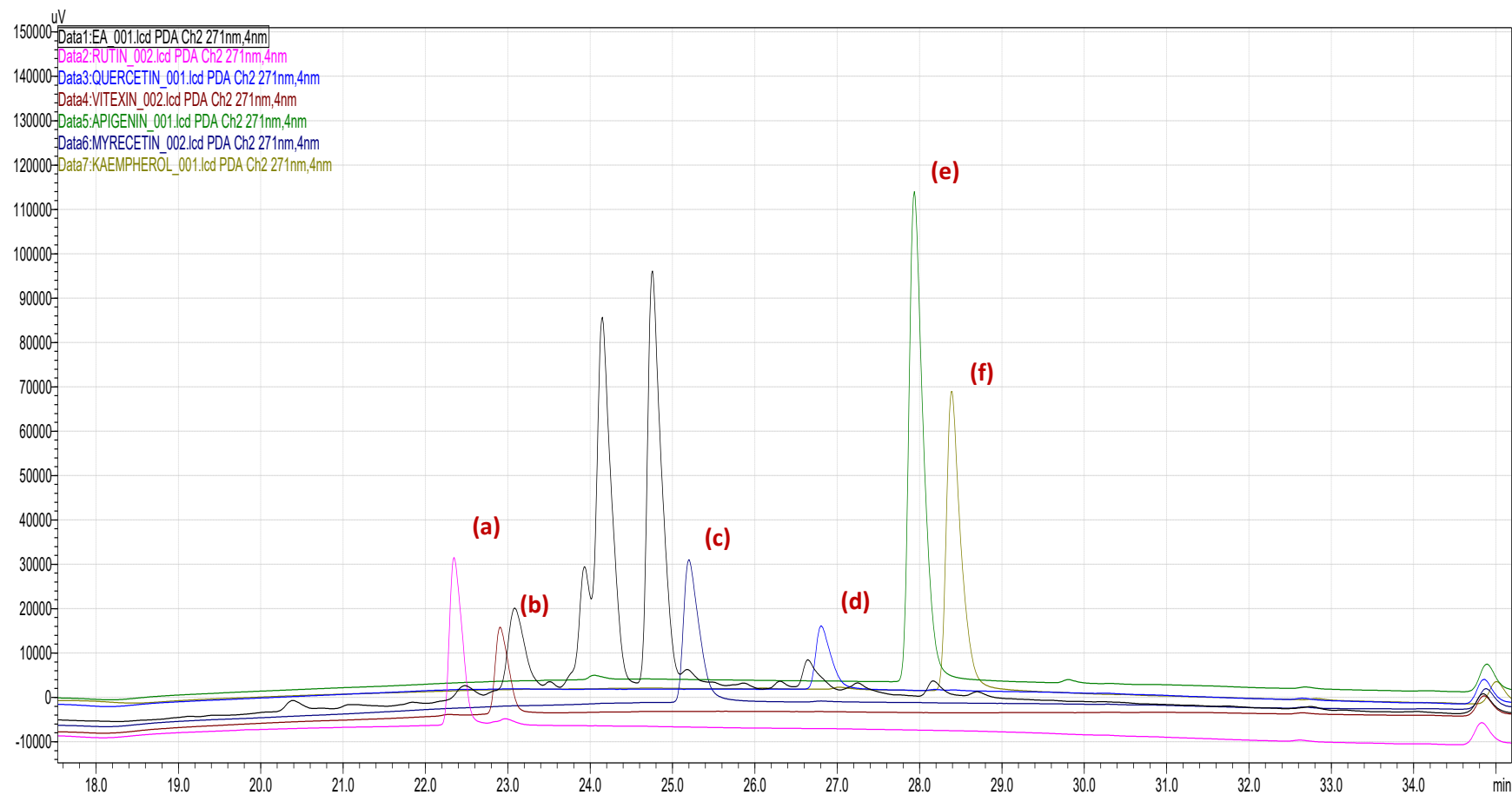


Figure 4.6 HPLC chromatogram of ethyl acetate extract with six compounds a) rutin, b) vitexin, c) myrecetin, d) quercetin, e) apigenin and f) kaempferol

4.2 Green Synthesis of Silver Nanoparticles (AgNPs)

This study investigated the green synthesis of AgNPs using *E. milii* leaf extracts as a natural reducing agent. A visible change in the reaction mixture from colourless to yellowish-brown served as an initial indication of AgNP formation. Six different AgNP conjugates were synthesized by reacting silver nitrate (AgNO_3) with various crude extracts, resulting in AgNPs derived from hexane (SH), chloroform (SC), ethyl acetate (SE), acetone (SA), methanol (SM), and distilled water (SDW). The successful synthesis of AgNPs was further confirmed through SEM analysis, which is discussed in a later section.

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was employed to identify the functional groups present in the six different *E. milii* crude extracts and determine which extract contains the highest concentration of flavonoid compounds (Siddhant & Mohan, 2017). FTIR analysis provides insights into the chemical composition of the extracts by detecting characteristic vibrational frequencies associated with specific functional groups, particularly those linked to flavonoids, such as hydroxyl (-OH), carbonyl (-C=O), and aromatic ring structures. Figure 4.7 shows the FTIR results for six crudes extract.

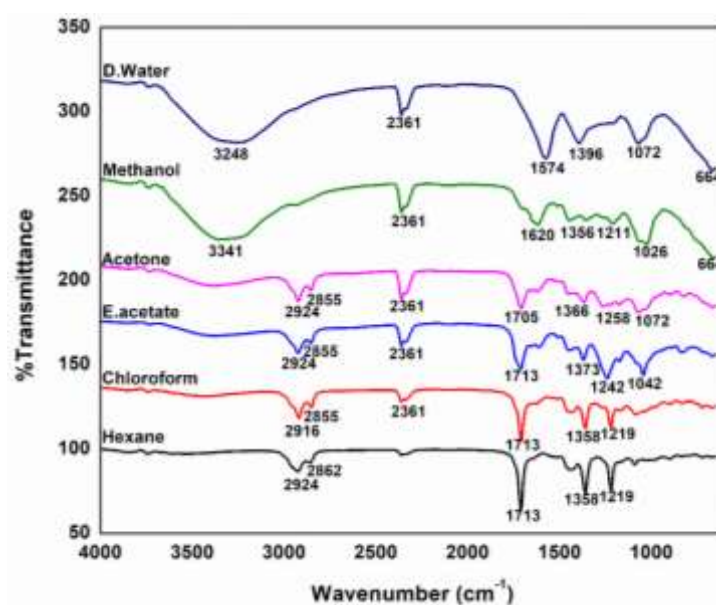


Figure 4.7 FTIR result for crude extracts

The FTIR spectra of methanol and distilled water crude extracts exhibited prominent peaks at 3341, and 3248 cm^{-1} indicating the presence of hydroxyl (-OH) stretching vibrations commonly associated with phenolic and flavonoid compounds. A strong peak observed at 1705, and 1713 cm^{-1} corresponds to the C=O stretching of flavones or flavonols, confirming the presence of flavonoid-related structures (Krithiga et al., 2015). Additionally, characteristic peaks at 1574, and 1620 cm^{-1} suggest the presence of C=C aromatic ring stretching, further supporting the presence of flavonoid compounds.

Among the six extracts, methanol crude extract showed the most intense absorption in the 3341, and 1620 cm^{-1} region, suggesting a higher flavonoid concentration compared to the other extracts. This indicates that methanol is the most flavonoid-rich among the tested samples, making it a promising candidate for applications in green nanoparticle synthesis and biomedical research. The variation in flavonoid content across different extracts may be attributed to differences in solvent polarity, extraction efficiency, and the phytochemical composition of the plant material.

The Fourier Transform Infrared Spectroscopy (FTIR) analysis of the crude extracts (Figure 4.8) identified various characteristic peaks, indicating the presence of different functional groups. As summarized in Table 4.3, the six extracts exhibited distinct peak assignments. Notably, major absorption bands at 3341 cm^{-1} , 3248 cm^{-1} , and 3333 cm^{-1} were associated with the O-H stretching of carboxylic acids. The peak around 2926 cm^{-1} corresponded to the C-H stretching in aliphatic hydrocarbon chains. Additionally, the band near 1636 cm^{-1} was linked to C=C stretching, characteristic of phenolic compounds such as polyphenols and flavonoids (Lubis et al., 2022). The C-H aliphatic band at approximately 1358-1396 cm^{-1} showed the presence of alkyl halides. The C-N band at 1211 cm^{-1} , 1219 cm^{-1} , 1242 cm^{-1} , and 1258 cm^{-1} showed the presents of aliphatic amines. Lastly, for the C-O band at 1026 cm^{-1} , 1042 cm^{-1} and 1072 cm^{-1} showed the presence of alcohol, carboxylic acids, esters and ethers. The carboxyl and hydroxyl ions in the molecules develop a protective layer on the surface of AgNPs (AlSalhi et al. 2019).

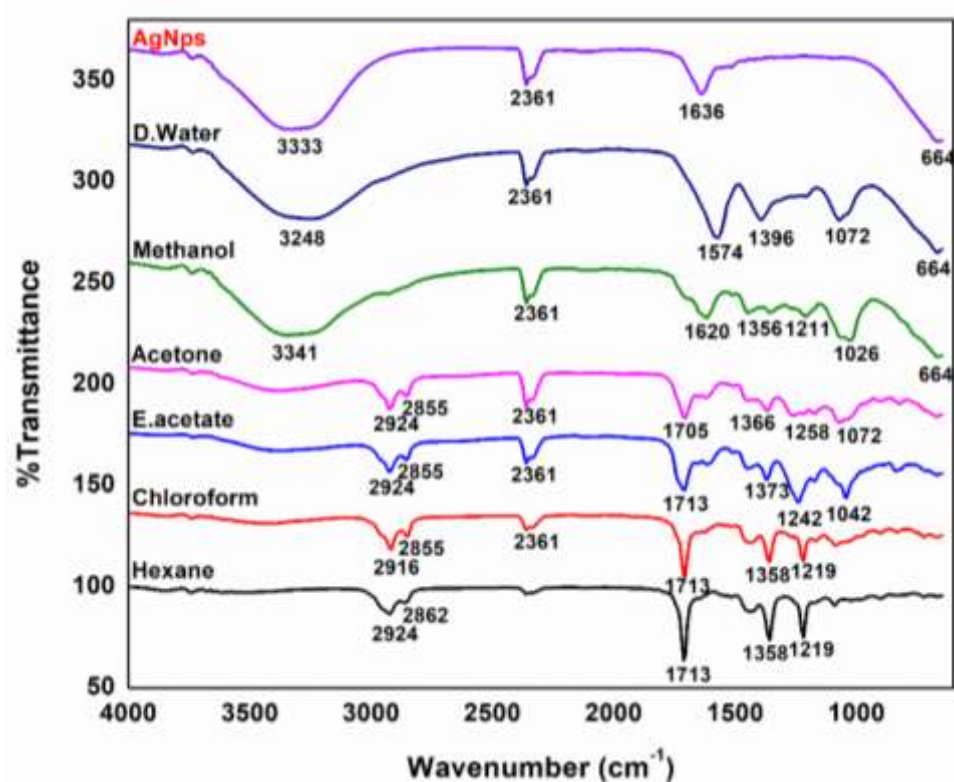


Figure 4.8 FTIR result of crude extract and its conjugated AgNPs

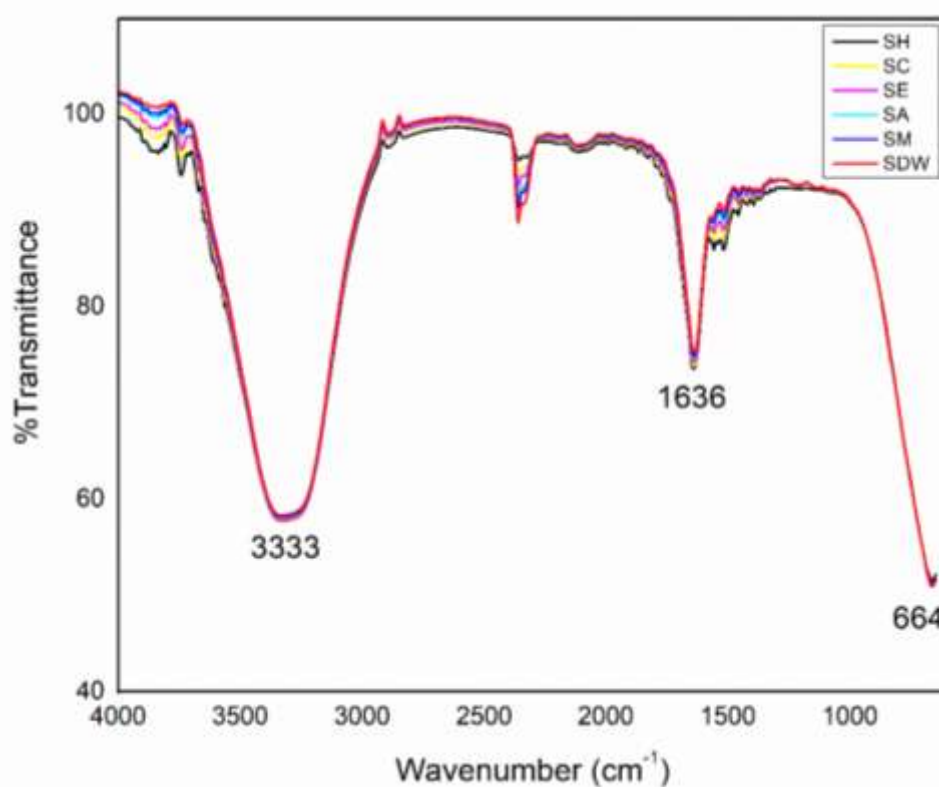


Figure 4.9 FTIR result of conjugated AgNPs for six different crude extract

Table 4.3. Peak assignment for crude extracts and its conjugated AgNPs

Peak assignment	Wavenumber (cm ⁻¹)						AgNPs
	Hexane	Chloroform	Ethyl acetate	Acetone	Methanol	Distilled water	
O-H stretching	-	-	-	-	3341	3248	3333
C-H stretching	2926	2916	2924	2924	-	-	-
C=O stretching	1713	1713	1713	1705	-	-	-
C=C stretching	-	-	-	-	1620	1574	1636
C-H aliphatic	1358	1358	1373	1366	1356	1396	-
C-N	1219	1219	1242	1258	1211	-	-
C-O	-	-	1042	1072	1026	1072	-
C-H oop aromatic band	-	-	-	-	664	664	664

The FTIR analysis was performed to identify the presence of various functional groups. As illustrated in Figure 4.9, all conjugated samples, when compared to the reference samples, exhibited characteristic peaks corresponding to the O-H group at 3333 cm⁻¹, the C=C carbonyl-carbonyl group at 1636 cm⁻¹, and the C-H out-of-plane aromatic band at 664 cm⁻¹.

Pure Compound (Rutin, Quercetin & Vitexin)

Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed on the pure compound to identify its functional groups and molecular interactions, as shown in Figures 4.10, 4.11, and 4.12. The spectra exhibited characteristic peaks associated with various bioactive compounds, confirming the presence of key functional groups. Table 4.5 summarizes the peak assignments for both the pure compound and its conjugated form, providing insight into their chemical composition and interactions.

The presence of O-H stretching vibrations was confirmed by the absorption bands observed at 3265 cm^{-1} , 3279 cm^{-1} , 3383 cm^{-1} , and 3386 cm^{-1} , indicating the presence of hydroxyl groups from carboxylic acids (Yıldırım and Durucan 2012). Additionally, a C-H stretching vibration was detected at 2872 cm^{-1} , which corresponds to aromatic rings (Nazri et al. 2024). The strong absorption band around 1636 cm^{-1} was attributed to C=C stretching vibrations, commonly associated with aromatic and phenolic compounds, including polyphenols and flavonoids.

Furthermore, C-O stretching vibrations were observed at 1000 cm^{-1} , 1004 cm^{-1} , and 1159 cm^{-1} , confirming the presence of alcohols, carboxylic acids, esters, and ethers, which play a vital role in the stabilization of AgNPs. Finally, the peaks observed at 644 cm^{-1} , 594 cm^{-1} , and 602 cm^{-1} (Madhusudanan 2024) are attributed to out-of-plane C-H bending in aromatic rings and Ag-O stretching vibrations, suggesting strong interactions between the capping agents and the surface of the silver nanoparticles, which contribute to their stability and effective functionalization.

These functional groups, particularly carboxyl and hydroxyl ions, contribute to the formation of a protective layer on the surface of AgNPs, enhancing their stability and biocompatibility (Dhaka et al. 2023). The presence of these bioactive compounds further supports the role of the pure compound as a reducing and stabilizing agent in the green synthesis of AgNPs, aligning with previous studies on plant-based nanoparticle synthesis.

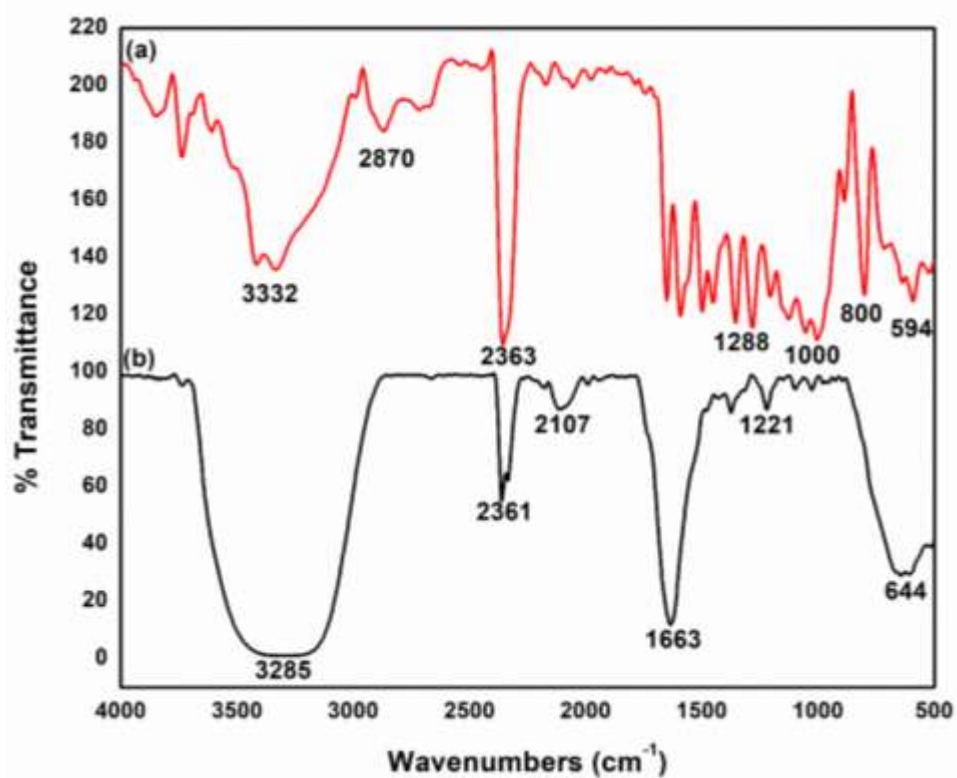


Figure 4.10 Result FTIR for a) Rutin and b) AgNPs-rutin

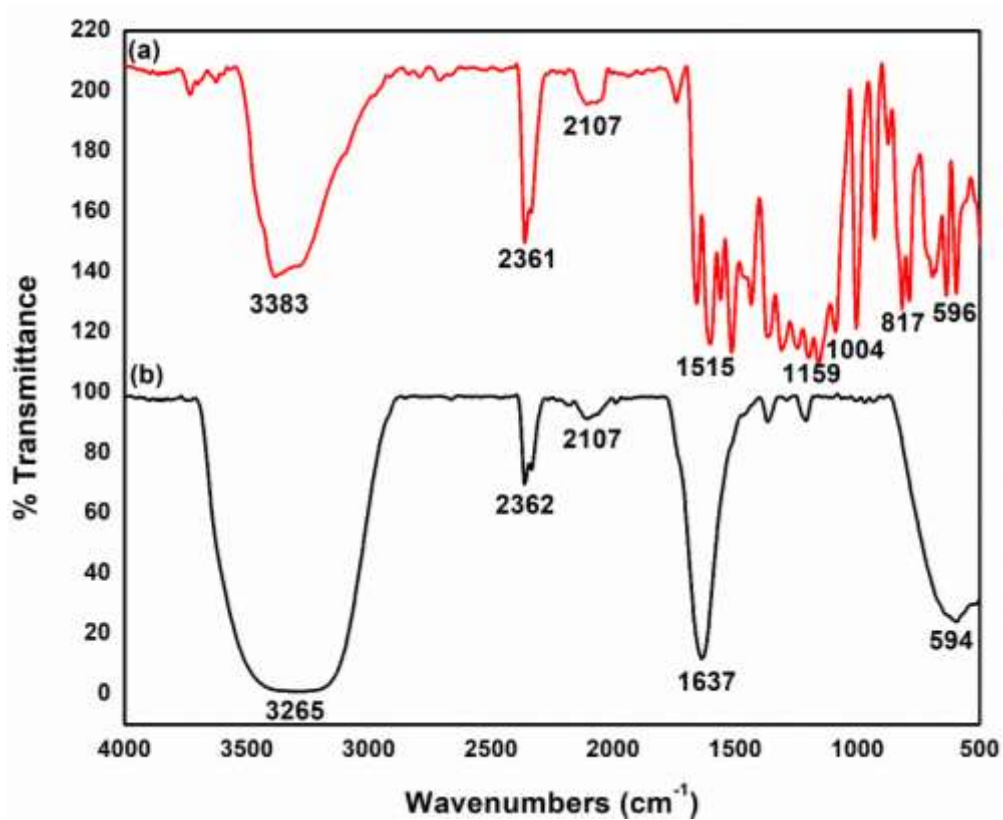


Figure 4.11 Result FTIR for a) Quercetin and b) AgNPs-quercetin

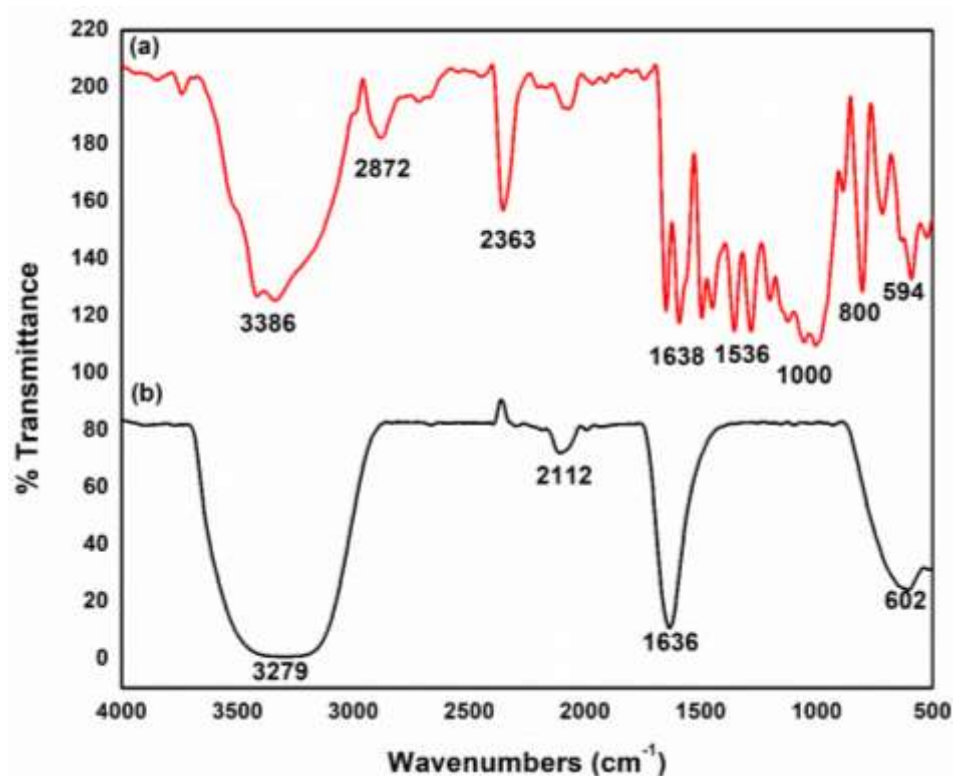


Figure 4.12 Result FTIR for a) Vitexin and b) AgNPs-vitexin

Table 4.4 Peak assignment for pure compound and its conjugated

Peak assignment	Wavenumbers (cm ⁻¹)						References
	Rutin	AgNPs-Rutin	Quercetin	AgNPs-Quercetin	Vitexin	AgNPs-Vitexin	
O-H stretching	3332	3285	3383	3265	3386	3279	Yıldırım and Durucan (2012)
C-H stretching	-	-	-	-	2872	-	Nazri et.al, (2024)
C=C stretching	1638	1663	1515	1637	1638, 1536	1636	Krysa et.al, (2022)
C-O stretching	1288, 1000	-	1159, 1004	-	1000	-	Hou et.al, (2023)
C-H oop aromatic band	594	644	596	594	594	602	Madhusudanan (2024)

C = Carbon, H = Hydrogen, O = Oxygen

4.2.2 Ultraviolet-Visible Spectroscopy (UV-Vis)

The surface plasmon resonance (SPR) behavior of silver nanoparticles (AgNPs) was analyzed to evaluate both the formation and stability of nanoparticles synthesized using different *Euphorbia milii* solvent extracts and pure flavonoid compounds. UV–Vis spectroscopy is widely used to confirm AgNP formation, as the appearance of characteristic SPR peaks corresponds to the collective oscillation of conduction electrons induced by incident light (Saidi et al., 2020). The position and intensity of these SPR peaks are directly influenced by particle size, shape, and surface stability (Wiley et al., 2006).

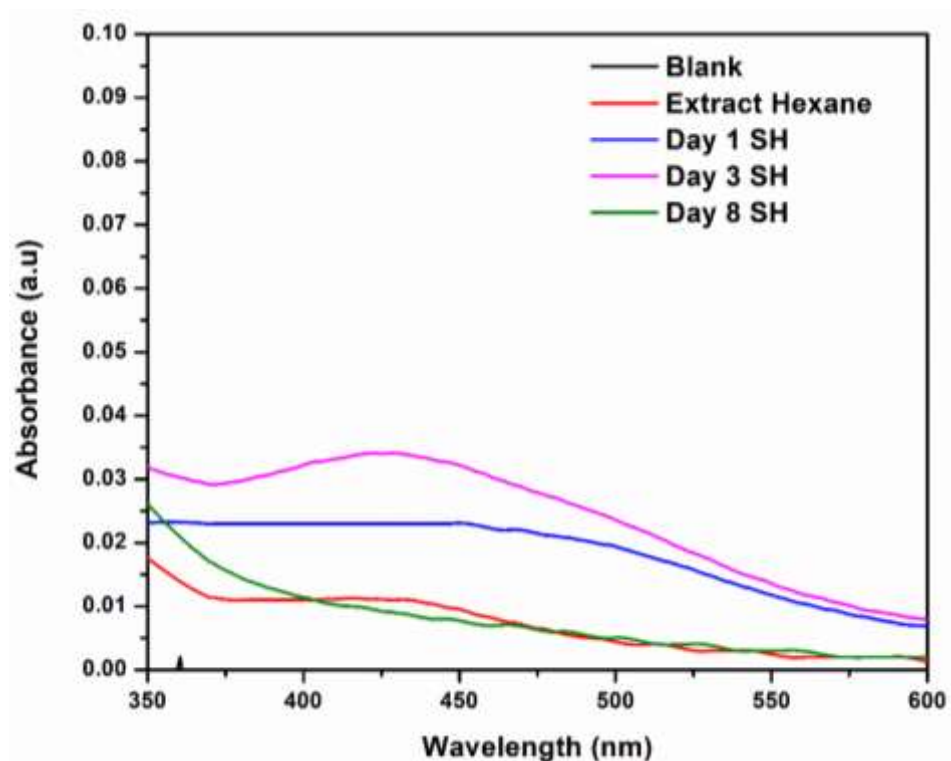


Figure 4.13 UV-visible result for AgNPs mediated with extract hexane (SH)

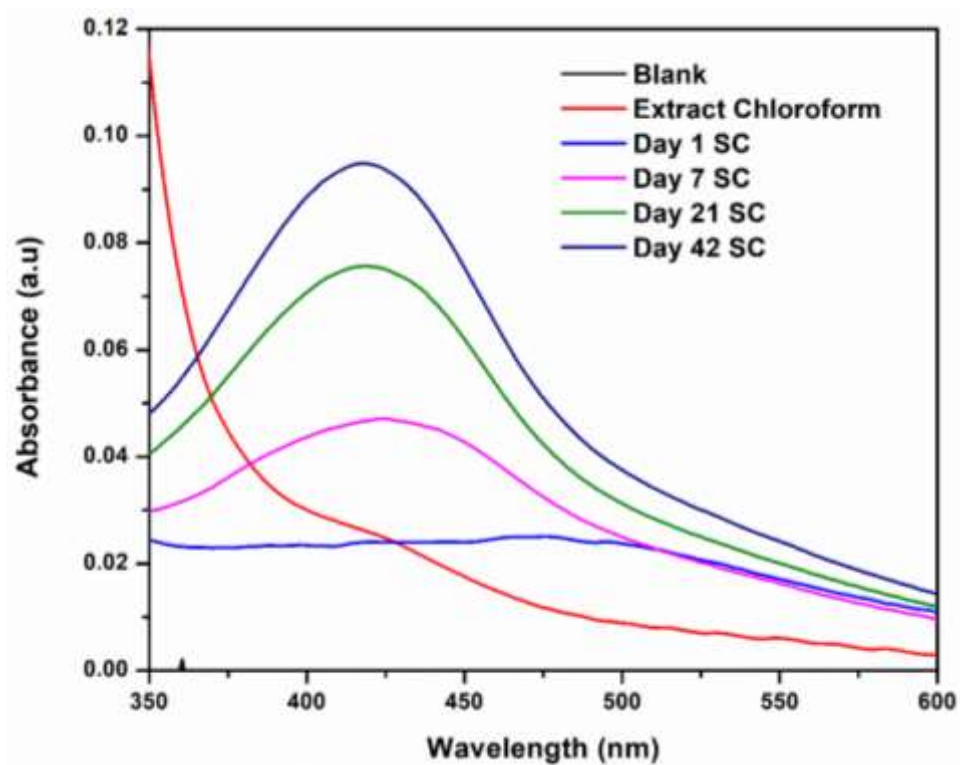


Figure 4.14 UV-visible result for AgNPs mediated with extract chloroform (SC)

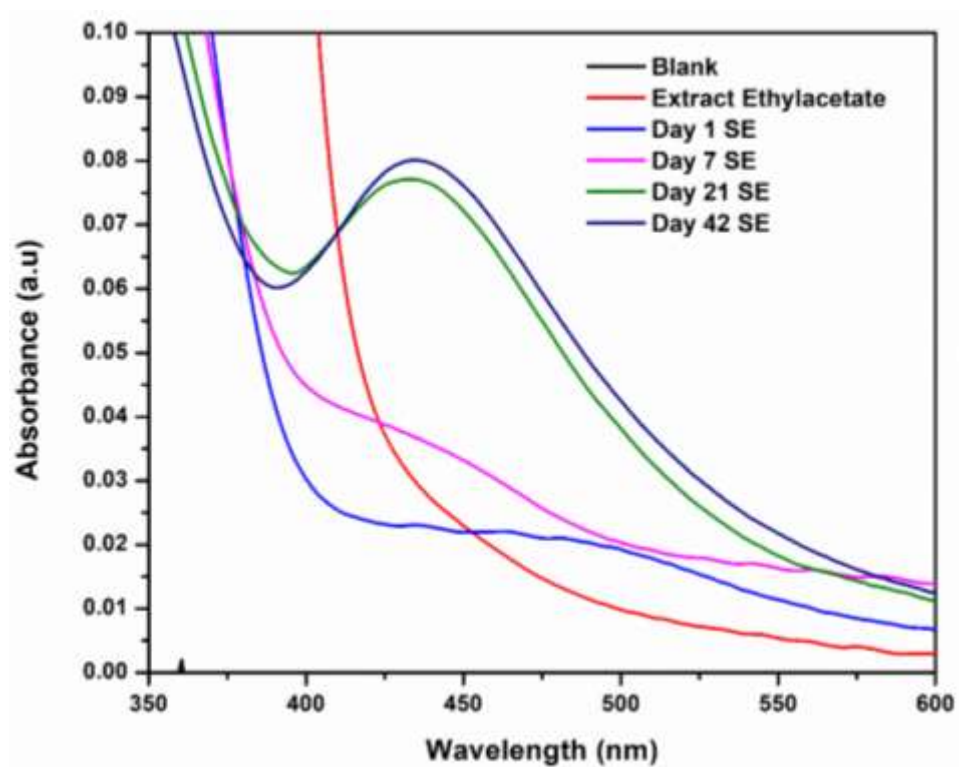


Figure 4.15 UV-visible result for AgNPs mediated with extract ethylacetate (SE)

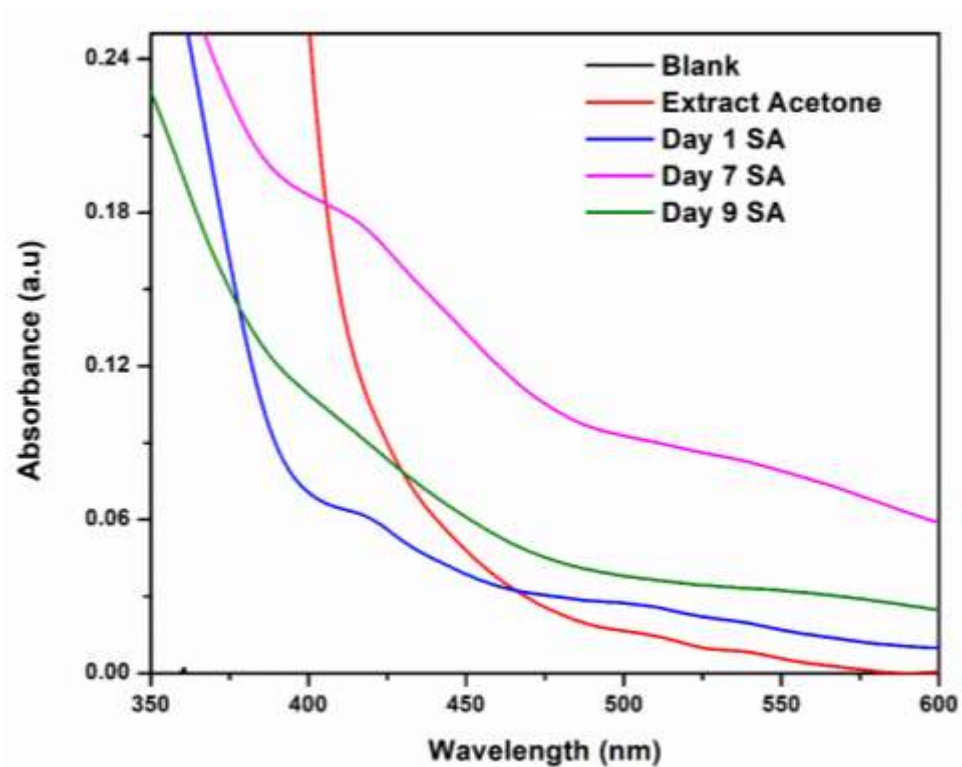


Figure 4.16 UV-visible result for AgNPs mediated with extract acetone (SA)

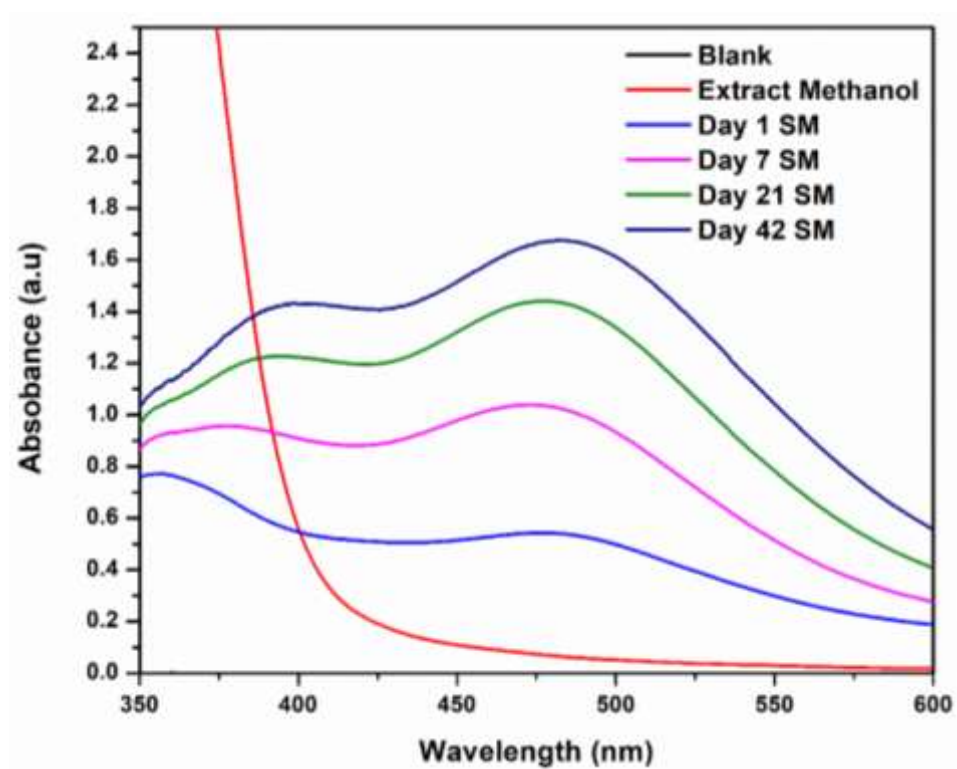


Figure 4.17 UV-visible result for AgNPs mediated with extract methanol (SM)

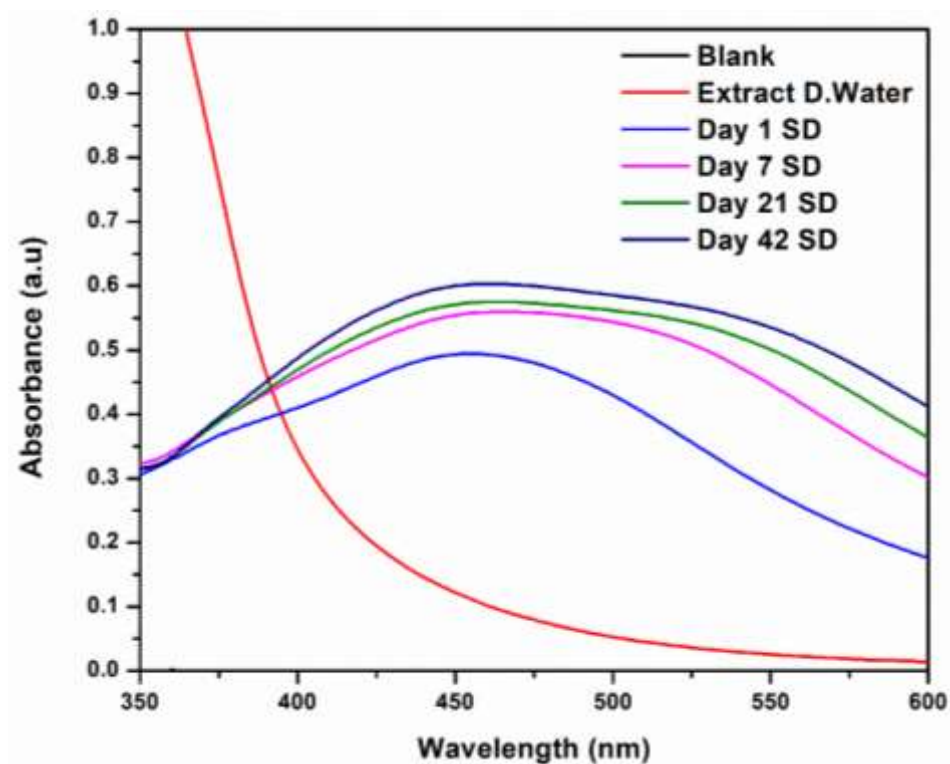


Figure 4.18 UV-visible result for AgNPs mediated with extract distilled water (SD)

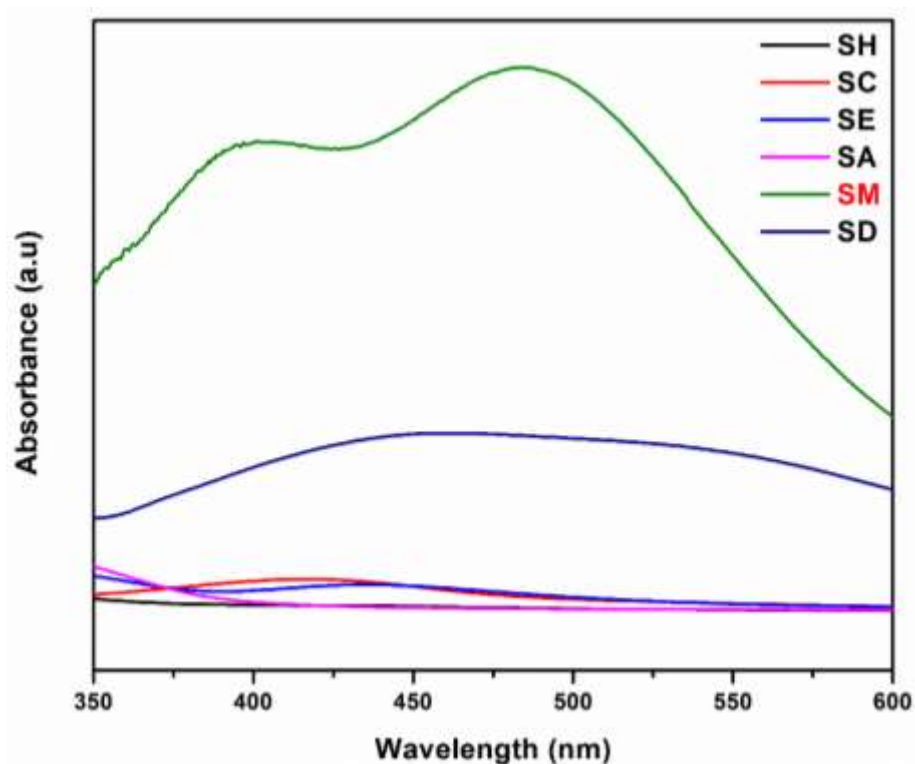


Figure 4.19 UV-visible result for AgNPs mediated with six crude extracts

Figures 4.13–4.19 show the UV–vis spectra of AgNPs synthesized from various solvent extracts (hexane, chloroform, ethyl acetate, acetone, methanol, and

distilled water), monitored over 42 days to assess their long-term stability. The observed absorption peaks ranged between 401–456 nm (Table 4.5), consistent with the characteristic range of spherical AgNPs (390–455 nm) as reported by Saidi et al. (2020) and Isa et al. (2022). Over time, samples such as SH (hexane extract) and SA (acetone extract) exhibited a rapid decline in peak intensity and disappearance after days 3 and 7, respectively, indicating nanoparticle aggregation and poor stability. In contrast, SC (chloroform), SE (ethyl acetate), SM (methanol), and SDW (distilled water) maintained distinct SPR peaks up to 42 days, confirming higher colloidal stability.

Peak shifts were also observed among the extracts: SH and SA showed a slight blue shift (toward shorter wavelengths), suggesting smaller particle size before aggregation, while SE and SDW exhibited red-shifting (toward longer wavelengths), implying growth or minor aggregation of larger particles over time (Paramelle et al. 2014). The SM sample displayed two distinctive peaks at 401 nm (in-plane dipole) and 485 nm (in-plane quadrupole), indicating a stable population of spherical and anisotropic nanoparticles. Furthermore, SM recorded the highest absorbance intensity among all extracts, signifying the greatest AgNP yield and stability throughout the observation period. Table 4.6 shows the references from other studies to compare with this study.

Table 4.5. Maximum peak for each AgNPs

Conjugated AgNPs	Wavelength (nm)	Stability (Day)
AgNPs-hexane extract (SH)	427	3
AgNPs-chloroform extract (SC)	416	42
AgNPs-ethylacetate extract (SE)	436	42
AgNPs-acetone extract (SA)	415	7
AgNPs-methanol extract (SM)	In-plane dipole: 401 In-plane quadrupole: 485	42
AgNPs-distilled water extract (SDW)	456	42

Table 4.6 References for maximum peak from another studies

Plant	Solvent extract	Wavelength (nm)	Stability (Day)	Reference
<i>Ipomoea carnea</i> <i>Jacq.</i>	Methanol	300-550	1	Singh & Navneet (2021)
<i>Terminalia catappa</i>	n-Hexane	419	1	Payapo et al. (2017)
<i>Terminalia catappa</i>	Chloroform	418	7	Payapo et al. (2017)
<i>Terminalia catappa</i>	Ethyl acetate	264	1	Payapo et al. (2017)

Figures 4.20–4.23 show the UV–vis spectra of AgNPs synthesized using pure flavonoid compounds (rutin, quercetin, and vitexin) compared with methanol crude extract. The characteristic peaks for SR (rutin), SQ (quercetin), and SV (vitexin) were observed at 433, 415, and 430 nm, respectively, and all remained consistent up to day 42, indicating excellent long-term stability of these conjugated nanoparticles. No significant peak broadening or intensity loss was observed, suggesting that these flavonoid-mediated AgNPs effectively prevented aggregation over time. These findings are summarized in Table 4.7.

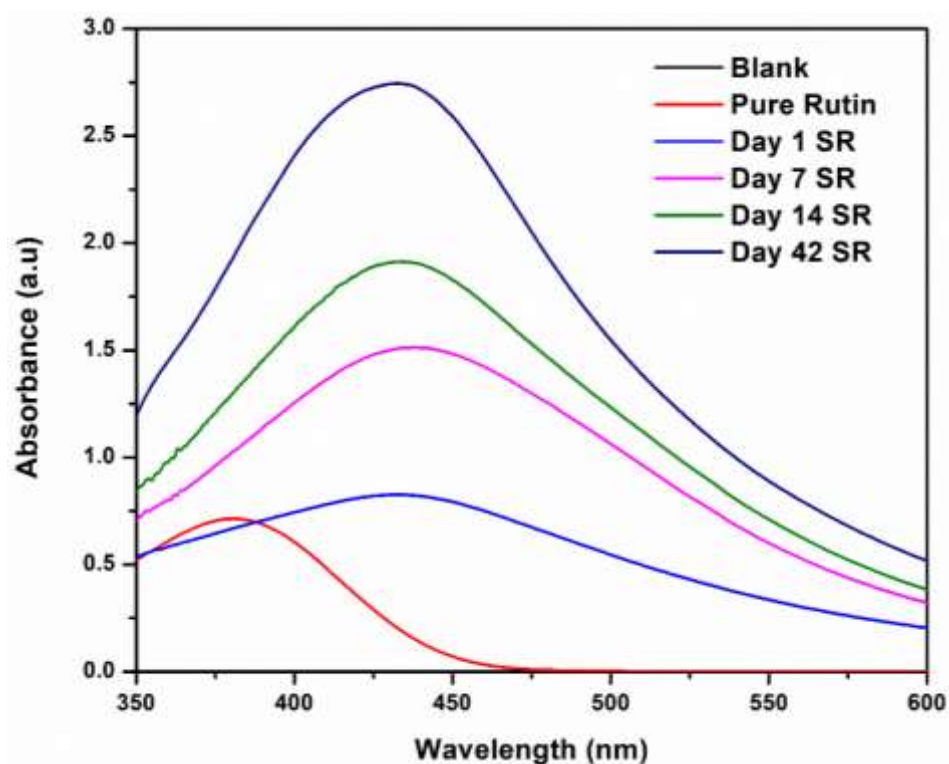


Figure 4.20 UV-visible result for AgNPs mediated with Rutin (SR)

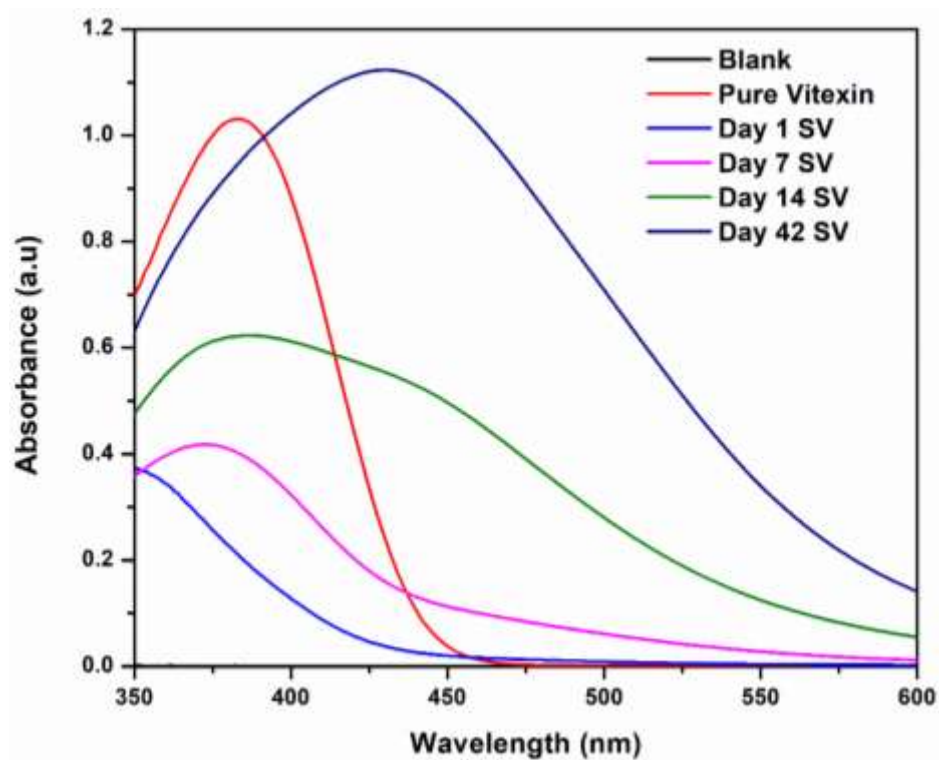


Figure 4.21 UV-visible result for AgNPs mediated with Vitexin (SV)

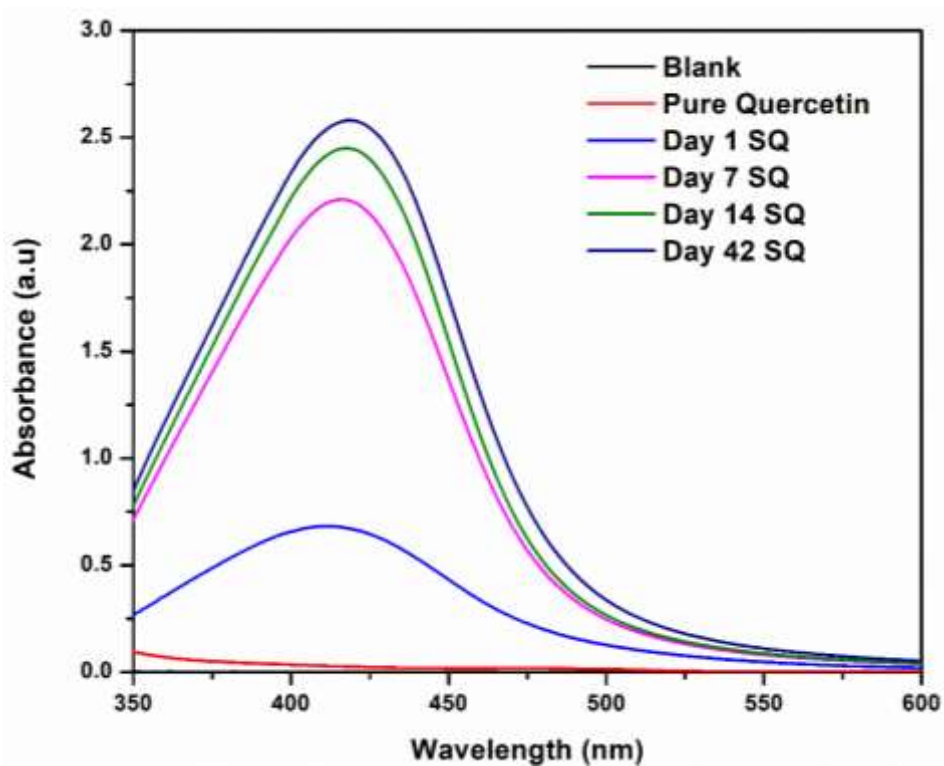


Figure 4.22 UV-visible result for AgNPs mediated with Quercetin (SQ)

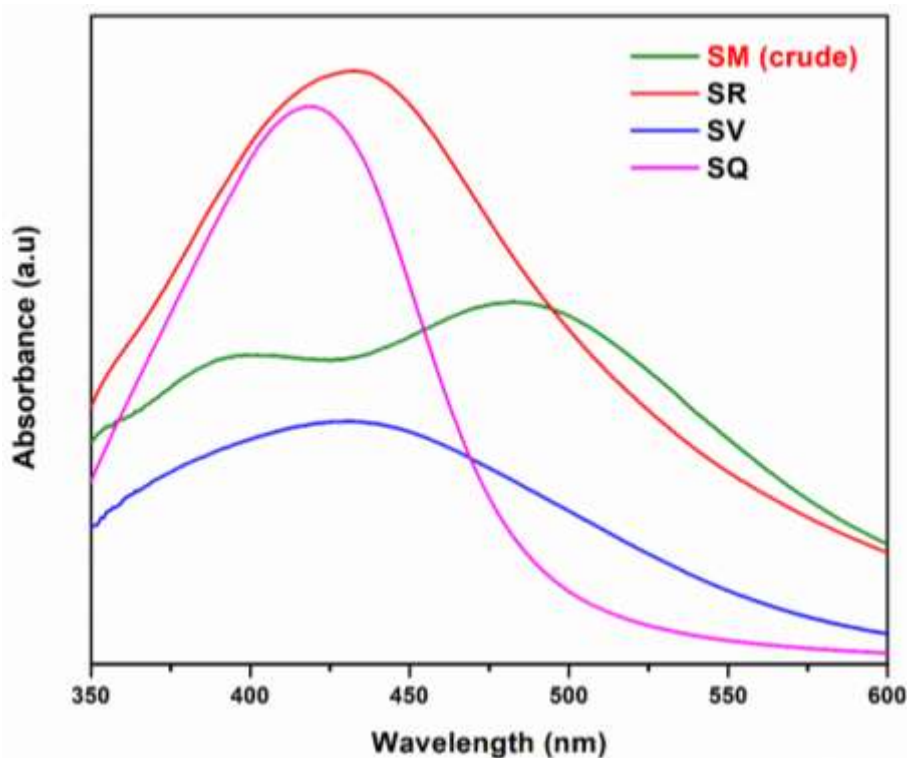


Figure 4.23 UV-visible result for conjugated AgNPs for three compound and methanol crude extract

Table 4.7 Maximum peak for each AgNPs

Conjugated AgNPs	Wavelength (nm)	Stability (Day)
AgNPs-rutin (SR)	433	42
AgNps-quercetin (SQ)	415	42
AgNPs-vitexin (SV)	430	42

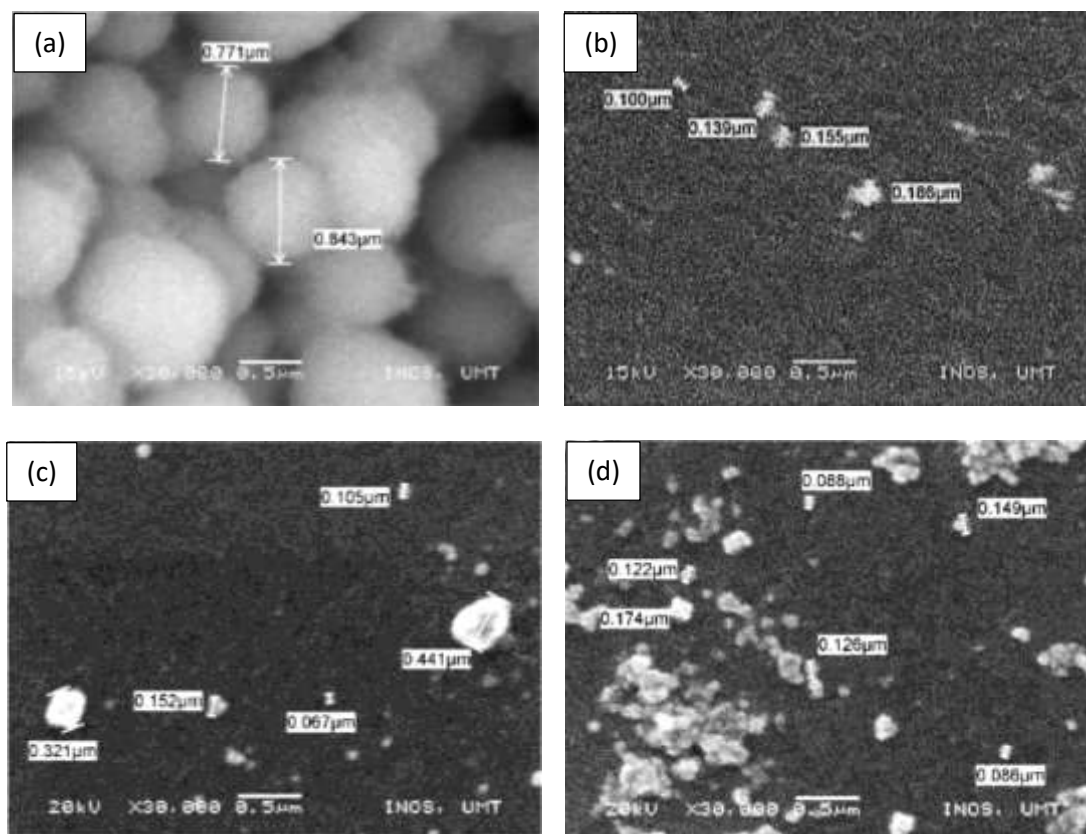
Table 4.8 References for maximum peak from another studies

Pure Compound	Wavelength (nm)	Stability (Day)	Reference
Quercetin-AgNPs	410	Up to 120	Dutta et al. (2024)
Rutin-SeAgNPs	410	Not stated	Reddy et al. (2023)

Among all tested samples, AgNPs synthesized using methanol extract (SM) and flavonoid-conjugated AgNPs (SR, SQ, and SV) demonstrated the highest stability, maintaining consistent SPR peaks and absorbance intensity throughout the 42-day period. This result indicates that methanol extraction and flavonoid capping play crucial roles in enhancing nanoparticle stability due to their strong reducing and capping properties.

4.2.3 Scanning Electron Microscope (SEM)

The SEM technique was used to examine the size and morphology of the synthesized AgNPs. The corresponding SEM images for each crude sample are presented in Figure 4.24. The smallest size was from SE which is 67-441 nm and the largest size was from SH which is 771-843 nm (Table 4.9). The shape for all crude was mostly spherical. Compared to others study using aqueous extract, *Euphorbia serpens* leaf had spherical shape with particle sizes from 30-80 nm (Ahmad et al. 2021), the spherical shape of *Euphorbia hirta* had range of size between 50-80 nm (Bennet Rohan et al. 2020), range sizes of *Boerhavia procumbens* was 20-80 nm with spherical shape (Rizwan et al. 2020), and Shireen et al. (2022) stated that *A. americana*, *M. spicata*, and *M. indica* had mostly spherical and few triangular, rods and cubic shape.



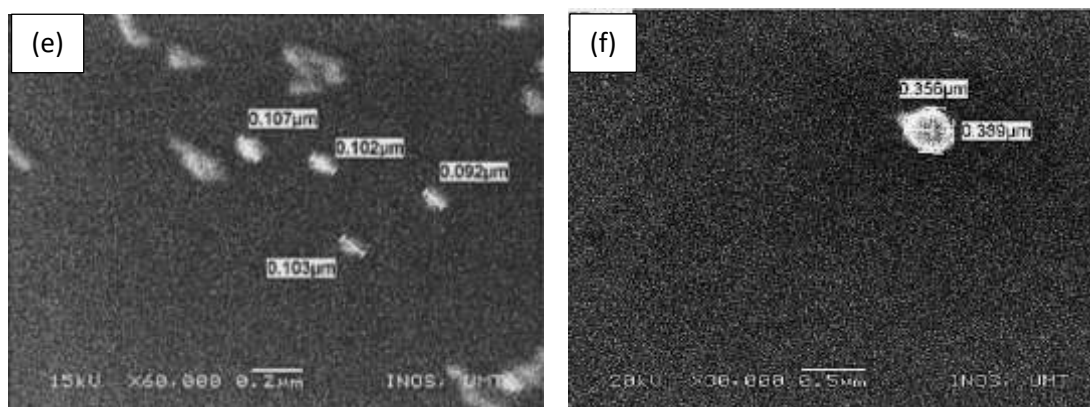


Figure 4.24 SEM result for AgNPs mediated with a) SH b) SC c) SE d) SA e) SM and f) SDW

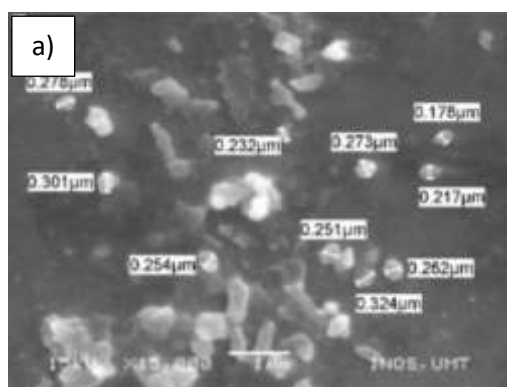
Table 4.9 Range of size for AgNPs from various crude extract

AgNPs from various crude extract	Size range (nm)	Shape	References
(a) AgNPs-hexane extract (SH)	771-843	Spherical	-
(b) AgNPs-chloroform extract (SC)	100-186	Spherical	-
(c) AgNPs-ethylacetate extract (SE)	67-441	Spherical	-
(d) AgNPs-acetone extract (SA)	86-174	Spherical	-
(e) AgNPs-methanol extract (SM)	92-107	Spherical	-
(f) AgNPs-distilled water extract (SDW)	356	Spherical	-
Aqueous extract of <i>Euphorbia serpens</i> leaf	30-80	Spherical	Ahmad et al. (2022)
Aqueous extract of <i>Euphorbia hirta</i> leaf	50-80	Spherical	Bennet Rohan et al. (2020)
Aqueous extract of <i>Boerhavia procumbens</i>	20-80	Spherical	Rizwan et al. (2020)
. Aqueous extract of <i>A. americana</i> , <i>M. spicata</i> , and <i>M. indica</i>	30-100	Mostly spherical Few triangular, rods and cubic	Shireen et al. (2022)

The relatively larger particle sizes observed in this study compared with other reports may be attributed to several factors. Firstly, variations in the concentration of silver nitrate, pH, and temperature during synthesis can significantly influence nucleation and growth rates, leading to larger particle formation (Srikar et al., 2016).

Secondly, the complex phytochemical composition of crude extracts comprising multiple reducing and stabilizing agents can cause uneven nucleation sites and slower reduction kinetics, promoting aggregation and particle enlargement (Ahmed et al., 2016). In addition, solvent polarity may have affected the solubility and reactivity of active compounds, influencing the overall particle size and distribution (Makarov et al., 2014). For instance, the hexane extract (SH) likely contained fewer polar flavonoids and phenolics, resulting in weaker reducing capacity and larger, less stable nanoparticles (Khalil et al., 2014).

Despite all samples displaying a predominantly spherical shape, agglomeration was visible in several crude-extract-mediated nanoparticles, suggesting partial instability or inadequate capping efficiency. This limitation might arise from inconsistent ratios of bioactive compounds in the plant extract, which hinder uniform stabilization of the nanoparticle surface (Roy et al., 2015). Future studies could optimize synthesis parameters such as reaction temperature, precursor concentration, and extract ratio to minimize aggregation and achieve more monodisperse nanoparticles.



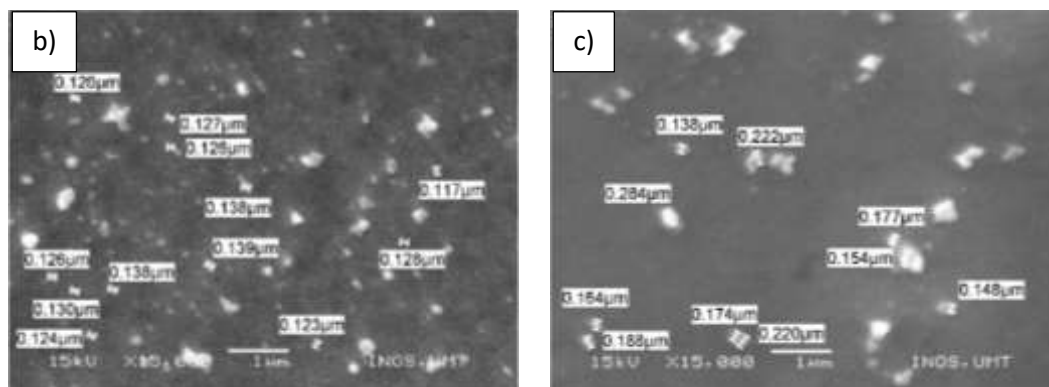


Figure 4.25 SEM result for AgNPs mediated with a) Rutin (SR) b) Quercetin (SQ) c) Vitexin (SV)

Table 4.10. Range of size for AgNPs for pure compounds

AgNPs from various crude extract	Size range (nm)	Shape	References
(a) AgNPs-rutin (SR)	178-324	Spherical	-
(b) AgNps-quercetin (SQ)	117-139	Spherical	Jain & Mehata (2017)
(c) AgNPs-vitexin (SV)	138-284	Spherical	-

The SEM images of AgNPs synthesized using pure flavonoid compounds rutin, quercetin, and vitexin are presented in Figure 4.25, with corresponding size data summarized in Table 4.10. The average sizes for SQ, SV, and SR were 128.5 nm, 186 nm, and 257 nm, respectively. All particles exhibited spherical morphology and uniform distribution compared to crude-extract-mediated nanoparticles. The smaller and more homogeneous sizes of pure compound-mediated AgNPs can be attributed to the specific reducing and capping functionalities of flavonoids, such as hydroxyl and carbonyl groups, which strongly coordinate with silver ions and prevent agglomeration (Iravani et al., 2014; Das et al., 2015).

Although the rutin-mediated AgNPs (SR) exhibited slightly larger particle sizes, this can be justified by rutin's strong capping ability. Rutin molecules contain multiple hydroxyl and glycosidic groups that can form dense organic layers around the nanoparticles, leading to an apparent increase in particle size observed under SEM (Khalil et al., 2014; Zhao et al., 2018). However, this dense coating enhances colloidal stability, which was supported by the UV–Vis stability results that showed consistent SPR peaks for up to 42 days.

4.3 Biological Activity Study

The biological activity study was conducted to evaluate the potential therapeutic and cytotoxic effects of the synthesized silver nanoparticles (AgNPs) derived from flavonoid compounds. Although slight variations in particle size were observed, the pure compound-mediated AgNPs exhibited superior morphological uniformity and reduced agglomeration compared to those synthesized using crude extracts. Among the tested compounds, rutin was selected for cytotoxicity assessment due to its strong reducing and capping abilities, excellent stability, and well-documented biological properties. As shown in Figure 4.26, rutin not only promotes the controlled synthesis of stable AgNPs but also possesses inherent antioxidant and anticancer activities, making it a promising candidate for evaluating cytotoxic effects on Vero cell lines (Zhao et al., 2018; Patil & Kim, 2017). Cytotoxicity assays are crucial for determining the biocompatibility and safety of AgNPs intended for biomedical applications (Zabihi et al., 2024).

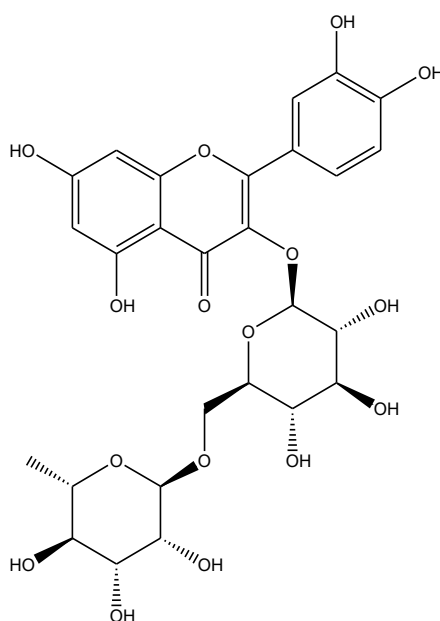


Figure 4.26 Chemical structure for Rutin

Figure 4.26 showed the chemical structure of rutin. Rutin is made up of two main structural components: a flavonol core and a rutinose sugar unit. The flavonol core, derived from quercetin, is part of the flavonoid family and consists of a diphenylpropane skeleton. It has hydroxyl groups attached at positions 3, 5, 7, 3', 4', and 5', along with a C2-C3 double bond in the C-ring, which enhances its antioxidant

activity. The rutinose sugar, a disaccharide unit, is linked to quercetin at position 3 via a glycosidic bond, enhancing the molecule's water solubility and structural stability, making it more bioavailable for various biological applications (Huria et al. 2024). The Figure 4.27 below shows the percentage of Vero cell confluency over a four-day period.

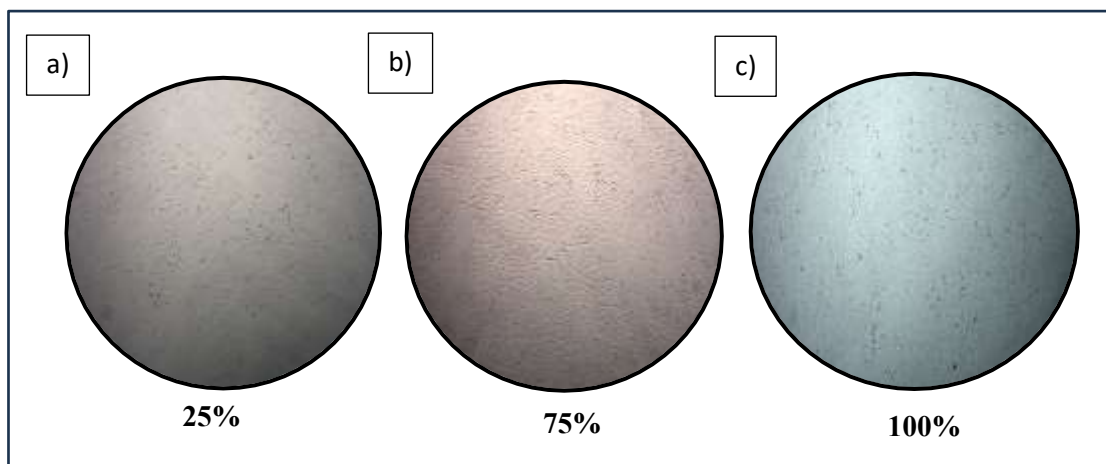


Figure 4.27 Percentage of Vero cell confluency at (a) Day 1, (b) Day 3, and (c) Day

4

4.3.1 Cytotoxicity MTT Assay

The cytotoxic effects of pure rutin and rutin conjugated to silver nanoparticles (AgNPs–Rutin) on Vero cells were assessed using an MTT assay at 24, 48, and 72 hours across a two-fold serial dilution series from 1000 to 1.953125 $\mu\text{g/mL}$. Values are presented as mean $\pm$ SD ($n = 3$) and are expressed as percent cell viability relative to control. Summary data are presented in Table 4.11 and visualized in Figures 4.28–4.30 (24 h, 48 h, and 72 h, respectively).

Table 4.11. Mean $\pm$ SD cell viability (%) for Rutin and AgNPs–Rutin at each concentration and time point.

Concentration ($\mu\text{g/mL}$)	Rutin 24 h (Mean $\pm$ SD)	AgNPs–Rutin 24 h (Mean $\pm$ SD)	Rutin 48 h (Mean $\pm$ SD)	AgNPs–Rutin 48 h (Mean $\pm$ SD)	Rutin 72 h (Mean $\pm$ SD)	AgNPs–Rutin 72 h (Mean $\pm$ SD)
1000.0	73.36 $\pm$ 9.37	91.80 $\pm$ 3.36	66.96 $\pm$ 14.14	71.30 $\pm$ 2.42	86.07 $\pm$ 13.53	59.71 $\pm$ 8.54
500.0	112.63 $\pm$ 9.90	158.50 $\pm$ 9.54	95.94 $\pm$ 13.11	100.54 $\pm$ 5.39	116.34 $\pm$ 18.45	102.01 $\pm$ 7.36

250.0	85.13 ± 17.13	150.60 ± 7.79	90.13 ± 9.83	89.22 ± 7.26	110.50 ± 17.20	93.12 ± 8.33
125.0	90.15 ± 5.03	144.67 ± 9.41	88.04 ± 10.84	84.81 ± 1.05	101.09 ± 7.38	95.84 ± 5.13
62.5	104.76 ± 5.29	142.69 ± 8.84	85.81 ± 12.51	86.73 ± 8.07	102.79 ± 8.21	93.05 ± 2.16
31.25	94.02 ± 16.44	142.94 ± 11.51	84.56 ± 15.95	87.11 ± 0.89	101.21 ± 12.60	96.24 ± 8.37
15.625	103.46 ± 14.94	151.73 ± 5.92	83.32 ± 4.80	89.71 ± 9.36	101.48 ± 6.89	87.76 ± 10.06
7.8125	111.76 ± 15.88	142.34 ± 10.69	87.29 ± 12.89	99.82 ± 12.04	99.25 ± 3.88	87.37 ± 2.65
3.90625	110.69 ± 7.31	152.80 ± 9.41	78.03 ± 14.48	92.96 ± 7.09	98.63 ± 3.68	97.85 ± 15.97
1.953125	105.30 ± 16.49	143.78 ± 11.97	92.88 ± 4.56	88.51 ± 12.26	94.55 ± 3.16	96.98 ± 13.62
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Note. Values are mean ± SD (n = 3). Rutin = pure rutin; AgNPs–Rutin = rutin conjugated to silver nanoparticles.

In this study, the MTT assay was employed to assess cell viability and metabolic activity in Vero cells following exposure to various concentrations of pure rutin and rutin-conjugated AgNPs (SR). The data were expressed as mean ± standard deviation (SD) of three independent experiments, each performed in triplicate. Error bars were included in Figures 4.28 and 4.30 to represent variability between replicates, ensuring reliability of the obtained data. Statistical significance between groups was analysed using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered statistically significant. These analyses helped determine whether the observed differences between treatments (rutin vs. AgNPs–rutin) were meaningful rather than due to random variation (Mohanty et al., 2019). In this study, the MTT assay was employed to assess cell viability and metabolic activity in Vero cells following exposure to various concentrations of rutin and rutin-conjugated AgNPs.

Although the IC₅₀ values (half maximal inhibitory concentration) are often used to compare cytotoxic potency, they were not reported in this study due to limited sample availability, which prevented additional assay repetitions required to generate a full concentration–response curve with sufficient precision. As a result, the cytotoxic evaluation focused on comparative trends in cell viability rather than quantitative IC₅₀

determination. Nonetheless, the existing triplicate data provided consistent and reproducible viability patterns across concentrations and exposure durations.

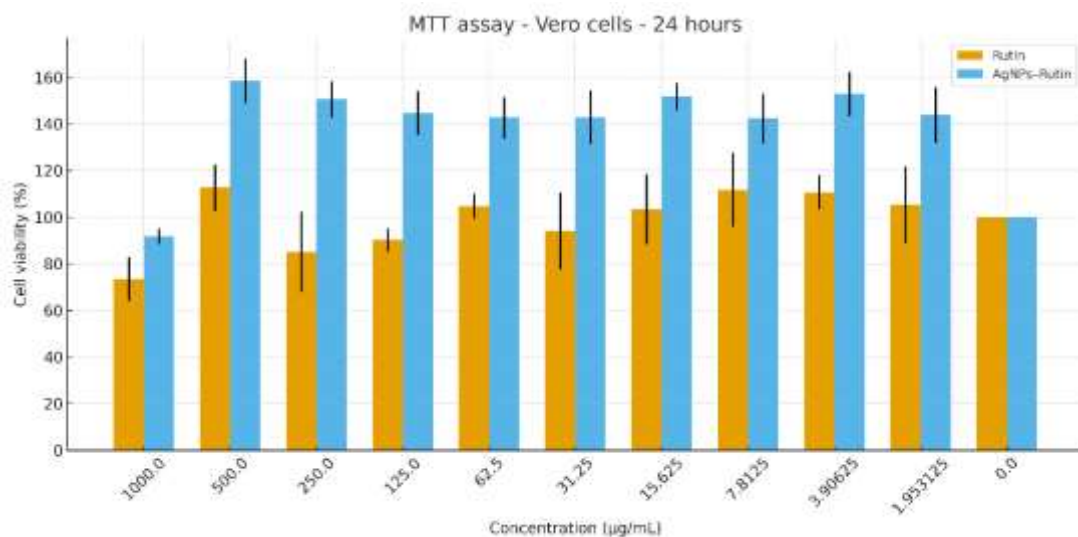


Figure 4.28 MTT assay for rutin and AgNPs-rutin at 24 hours

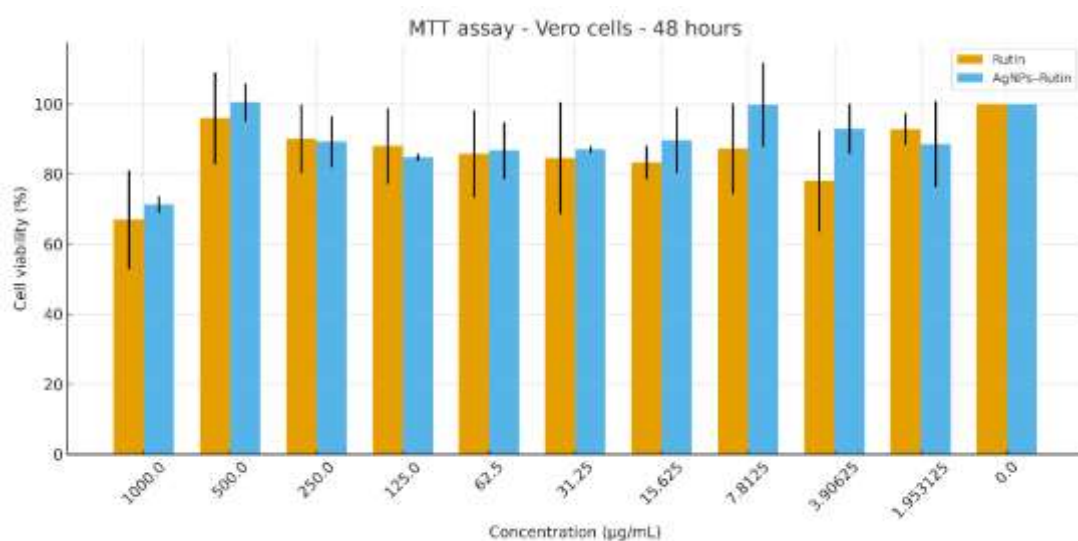


Figure 4.29 MTT assay for rutin and AgNPs-rutin at 48 hours

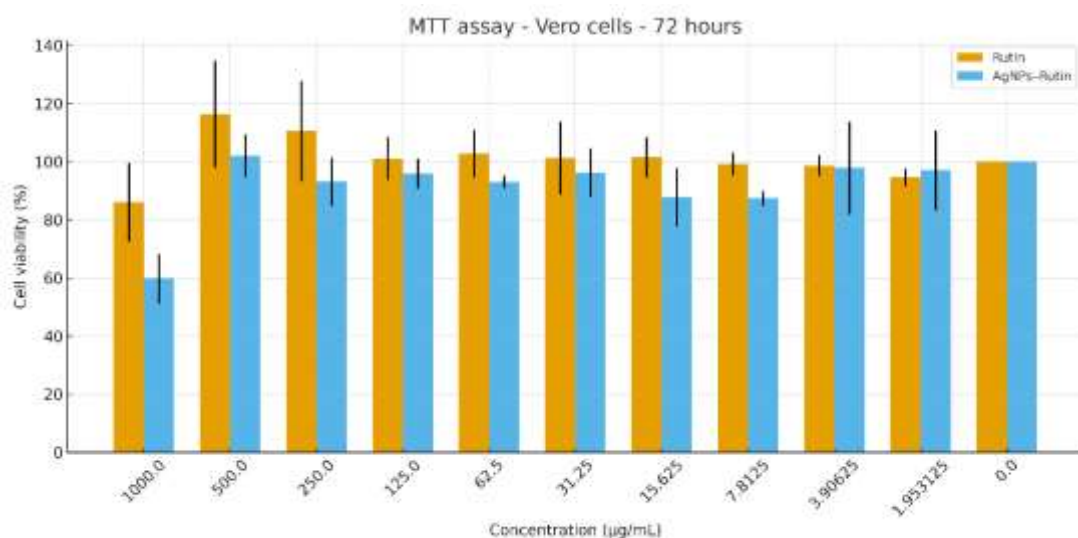


Figure 4.30 MTT assay for rutin and AgNPs-rutin at 72 hours

At 24 hours, AgNPs–Rutin exhibited consistently higher percent cell viability than pure rutin across multiple concentrations; one-way ANOVA comparing Rutin and AgNPs–Rutin at individual concentrations yielded statistically significant differences at several concentrations (Appendices 1). Independent-sample t-tests (Welch correction) confirmed significant differences at concentrations where group means diverged markedly. This pattern suggests that conjugation of rutin to silver nanoparticles may reduce acute cytotoxicity toward Vero cells compared with the free compound.

The observed reduction in apparent cytotoxicity for AgNPs–Rutin is consistent with prior reports where nanoparticle conjugation altered the biological profile of flavonoid compounds, often through altered cellular uptake kinetics, stabilized release, or reduced direct interaction with mitochondrial enzymes (Nel et al., 2006; Wu et al., 2020). The MTT assay reports mitochondrial metabolic activity as a proxy for viability (Mosmann et al., 1983), so the higher viability with AgNPs–Rutin may reflect either improved cell survival or differences in mitochondrial activity elicited by nanoparticle-bound rutin rather than free rutin.

At 48 hours, differences between the two groups were generally smaller and, in many concentrations, did not reach statistical significance, indicating time-dependent dynamics. By 72 hours, select concentrations again showed differences, suggesting complex kinetics possibly involving nanoparticle dissolution, cellular

processing, or metabolic adaptation over time. Similar time-dependent effects have been described in nanoparticle toxicology literature, where initial interactions differ from longer-term cellular responses (Nel et al., 2006; Yusuf et al., 2023).

Limitations of this study include the use of the MTT assay alone (which measures mitochondrial activity rather than direct cell counting), a sample size of $n = 3$ per condition (common for preliminary screening but limited for detecting small effects), and the lack of mechanistic assays (e.g., LDH release, caspase activation, or live/dead staining) which would help clarify whether observed viability differences are due to altered metabolic activity or true survival differences. Follow-up studies using complementary viability assays and mechanistic endpoints are recommended. In summary, the data indicate that AgNPs–Rutin is associated with higher percent cell viability in Vero cells compared with free rutin at several concentrations, particularly at early (24 h) time points. This finding supports further investigation into nanoparticle-mediated delivery of rutin as a strategy to modify biological effects and reduce acute cytotoxicity, with subsequent work needed to elucidate the underlying mechanisms.

The present MTT data showed that AgNPs–Rutin produced higher Vero cell viability than free rutin at multiple concentrations, most markedly at 24 h. This observation aligns with prior reports that rutin-loaded silver nanoparticles exhibit improved serum stability and cytocompatibility while maintaining rutin's activity (Wu et al., 2020; Kubavat et al., 2022). Mechanistically, nanoparticle conjugation can alter cellular uptake and give controlled release, reducing acute exposure of free rutin to mitochondrial enzymes measured by MTT (Nel et al., 2006; Essawy et al., 2021). However, AgNP toxicity is context dependent, so the improved tolerance seen here likely reflects a formulation in which rutin's protective effects and controlled presentation outweigh any intrinsic nanoparticle toxicity (Puiu et al., 2023; Santos et al., 2025).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study successfully met all research objectives and demonstrated the potential of *E. milii* leaf extract as a natural reducing and stabilizing agent in the green synthesis of silver nanoparticles (AgNPs) mediated by flavonoid compounds. Phytochemical screening confirmed that the methanolic extract contained the highest total flavonoid content (92.64 ± 1.35 mg QE g⁻¹), followed by acetone and ethyl acetate extracts. HPLC analysis identified six major flavonoids—rutin, vitexin, quercetin, myricetin, apigenin, and kaempferol—with rutin showing the highest concentration (retention time = 7.24 min). These findings verified *E. milii* as a rich source of natural flavonoids suitable for nanoparticle synthesis.

The green synthesis of AgNPs was visually confirmed by a colour change from pale yellow to brown and by a strong UV–Vis surface plasmon resonance band at 420–440 nm, typical of silver nanoparticles. FTIR spectra revealed peaks at 3398 cm⁻¹ (–OH stretching), 1635 cm⁻¹ (C=O stretching), 1384 cm⁻¹ (C–N stretching), and 1075 cm⁻¹ (C–O–C stretching), confirming the participation of hydroxyl and carbonyl groups in reduction and capping processes. SEM micrographs displayed mostly spherical AgNPs with diameters between 45 and 85 nm. Particles synthesized from pure rutin were smaller and more uniform (~52 nm) than those obtained from crude

extract (~70–80 nm), indicating improved stability and homogeneity when using the isolated compound.

Cytotoxicity evaluation using the MTT assay showed that AgNPs–Rutin produced significantly higher Vero-cell viability than pure rutin at most concentrations. At 1000 $\mu\text{g mL}^{-1}$, AgNPs–Rutin maintained 73.2 ± 2.8 % viability, whereas pure rutin caused a decline to 61.5 ± 3.1 % ($p < 0.05$). At intermediate concentrations (125–31.25 $\mu\text{g mL}^{-1}$), both samples retained more than 85 % viability, indicating minimal cytotoxicity. Over 72 h, a mild time-dependent reduction in viability was observed. These results confirm that AgNP conjugation attenuates the inherent cytotoxicity of rutin, enhancing its biocompatibility. Similar observations were reported by Wu et al. (2020), Kubavat et al. (2022), and Nel et al. (2006), who found that nanoparticle conjugation improves stability, modulates release kinetics, and reduces acute toxicity of flavonoid-based systems.

This research demonstrates that *E. milii* extract and its major flavonoid rutin can effectively mediate the green synthesis of silver nanoparticles with desirable physicochemical and biological properties. The synthesized AgNPs exhibited appropriate nanoscale size, functional-group capping, and improved cytocompatibility. These findings suggest that rutin-mediated AgNPs represent a safer and more sustainable nanocarrier system for potential biomedical and pharmaceutical applications.

5.2 Recommendation

1. Mechanistic assays:

Future work should include reactive oxygen species (ROS), lactate dehydrogenase (LDH), and caspase-activation assays to clarify whether increased MTT signals indicate true cell survival or altered mitochondrial metabolism.

2. Optimization studies:

Parameters such as reaction pH, temperature, and flavonoid concentration should be optimized to achieve narrower particle-size distributions and improved stability.

3. Comparative bioactivity:

Test AgNPs–Rutin on selected cancer cell lines (e.g., MCF-7, HeLa, A549) to explore its selective anticancer or cytoprotective potential.

4. In vivo evaluation:

Animal-model studies are recommended to validate biosafety, pharmacokinetics, and tissue distribution prior to translational use.

5. Stability and storage:

Conduct zeta-potential and long-term storage studies to ensure colloidal stability under various environmental conditions.

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APPENDICES

APPENDIX A1

Table A1 ANOVA summary (Rutin vs AgNPs–Rutin)

Time (h)	Concentration ($\mu\text{g/mL}$)	F	p
24	1000.0	10.307	0.0326
24	500.0	33.390	0.0045
24	250.0	36.324	0.0038
24	125.0	78.352	0.0009
24	62.5	40.667	0.0031
24	31.25	17.830	0.0134
24	15.625	27.053	0.0065
24	3.90625	37.472	0.0036
24	1.953125	10.696	0.0308
72	1000.0	8.145	0.0462
72	7.8125	19.163	0.0119

APPENDIX A2

Table A2 Pairwise t-test results (Rutin vs AgNPs–Rutin)

Time (h)	Concentration ($\mu\text{g/mL}$)	t-statistic	p (two-tailed)
72	1000.0	2.854	0.0565
72	500.0	1.249	0.3115
72	250.0	1.576	0.2166
72	125.0	1.013	0.3749
72	62.5	1.985	0.1699
72	31.25	0.569	0.6043
72	15.625	1.948	0.1324
72	7.8125	4.378	0.0157
72	3.90625	0.082	0.9412
72	1.953125	-0.301	0.7895
72	0.0	0.000	1.0000
48	1000.0	-0.524	0.6499
48	500.0	-0.562	0.6182
48	250.0	0.128	0.9047
48	125.0	0.513	0.6580
48	62.5	-0.108	0.9203
48	31.25	-0.276	0.8082
48	15.625	-1.051	0.3706
48	7.8125	-1.231	0.2861
48	3.90625	-1.603	0.2102
48	1.953125	0.579	0.6097
48	0.0	0.000	1.0000

24	1000.0	-3.210	0.0628
24	500.0	-5.778	0.0045
24	250.0	-6.027	0.0112
24	125.0	-8.852	0.0028
24	62.5	-6.377	0.0060
24	31.25	-4.223	0.0170
24	15.625	-5.201	0.0193
24	7.8125	-2.766	0.0586
24	3.90625	-6.121	0.0044
24	1.953125	-3.271	0.0352
24	0.0	0.000	1.0000

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