Enhanced Biological Activity of Biosynthesized Silver Nanoparticles Using Thymoquinone

By

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Department of Physics

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641 043

In partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN PHYSICS

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CERTIFIED AS A BONAFIDE RESEARCH WORK

12/05/23

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CHAPTER – I

INTRODUCTION

1.Introduction

Silver nanoparticles (AgNPs) have many potential applications in several fields, such as industrial, biomedical application and broad-spectrum antibacterial characteristics. Plant extract mediated synthesis of metal nanoparticles has an edge over microbial mediated biosynthesis of nanoparticles because the green synthesis of nanoparticles takes place extracellular. Plant extracts may act both as reducing agents and stabilizing agents in the synthesis of nanoparticles.

Thymoquinone (TQ) is one of the major bioactive molecules of *Nigella Sativa* seeds, which are responsible for the reduction of silver ions to AgNPs and subsequent capping of the formed particles with less toxicity.

1.1 Quinones in Nigella Sativa

Many active compounds have been identified in Nigella Sativa seeds. Figure 1.1 shows the most active compounds which are Dithymoquinone, Thymohydroquinone, Thymol and Thymoquinone **(Camila Spereta Bertanha et al., 2014).**

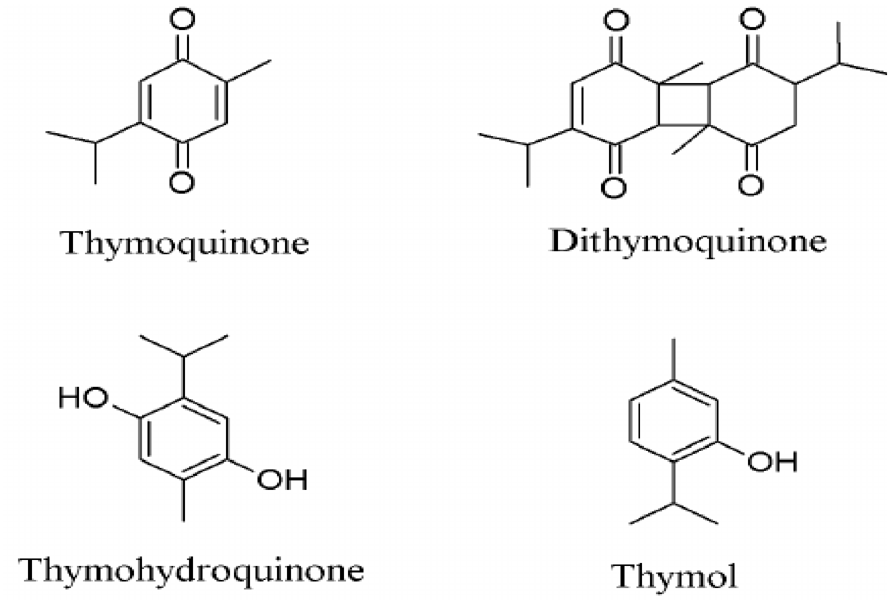

Figure 1.1: Active components in Nigella Sativa seeds

1.1.1 Dithymoquinone

Dithymoquinone(4b,8b-Dimethyl-3-7-di(propan-2-yl)-4a,8a-dihydrobiphenylene-1,4,5,8-tetron) is a bioactive isolate of Nigella Sativa. Chemically, it is a dimer of thymoquinone.

1.1.2 Thymohydroquinone

Thymohydroquinone (1,4-benzenediol, 2-methyl-5-(1-methylethyl)) is a component of black cumin seed oil and thyme. It is also a reduction product of thymoquinone which is a phytochemical compound found in Nigella Sativa plant. Thymoquinone exhibits antioxidant and analgesic properties.

1.1.3 Thymol

Thymol (2-isopropyl-5-methyphenol 5- methyl-2-isopropylphenol) is a phenol and had cymene derivative naturally.

1.1.4 Thymoquinone (TQ)

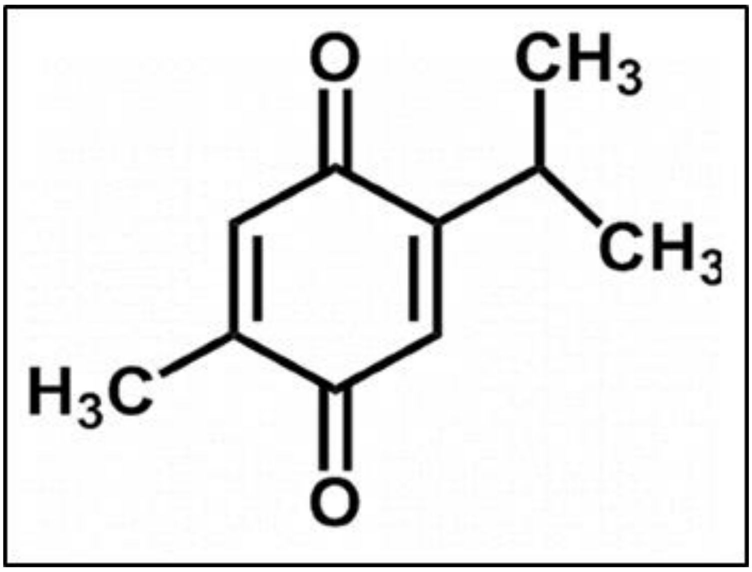

Figure 1.2: Structure of Thymoquinone

Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone) is a yellow crystalline monoterpene molecule which has a basic quinone structure with methyl and isopropyl groups attached to 2 and 5 positions of benzene ring in which a para substituted dione is conjugated. Thymoquinone has a chemical formula $C_{10}H_{12}O_2$.With successful extraction and identification of TQ from the Nigella sativa seeds, a number of studies have highlighted its therapeutic effects. These studies showed that TQ possess anti-inflammatory, anti-oxidant and anti-microbial activities **(Chen et al., 2016)**. Furthermore, a large body of evidence indicates that TQ has a very few side effects and no serious toxicity. TQ is known to be the primary active constituent of Nigella sativa seeds and it also showed promising results in the treatment of cancer. This dietary phytochemical study has received a lot of attention with an increasing interest in investigating it in preclinical and clinical studies to assess its health benefits. TQ has been linked to a variety of therapeutic benefits including bronchial asthma, dysentery, asthma, eczema, hypertension and obesity **(Bagchi K et al., 1998)**.

1.2 Metal Nanoparticles

Metallic nanoparticles or metal nanoparticles, a new terminology has been originated in the field of nanoparticles in recent few years. The noble metal like gold, silver, and platinum having beneficial effects on health are utilized for the synthesis of nanoparticles and designated as metallic nanoparticles. Nowadays researchers are focusing on metal nanoparticles, nanostructures and nanomaterial synthesis because of their conspicuous properties that are useful for catalysis, composite like polymer preparations, disease diagnosis and treatment, sensor technology, and labelling of optoelectronic recorded media. Different physical and chemical methods such as electrochemical changes, chemical reduction, and photochemical reduction are commonly employed for the preparation and stabilization of metallic nanoparticles. The selection of preparation method of metallic nanoparticle is equally important because during nanoparticle synthesis processes such as kinetics of interaction of metal ions with reducing agent, adsorption process of stabilizing agent with metal nanoparticles and various experimental techniques produces strong influence on its morphology (structure and size) stability and physicochemical properties. Gold is widely used in the medicines and Ayurvedic preparations in India and China. Gold nanoparticles are employed for many diagnostics and drug delivery purposes. Apart from this, other metal nanoparticles like silver nanoparticles are also employed for various biomedical applications such as separation science and novel drug delivery system. Silver is well known for its antimicrobial and inflammatory potential. This property is selectively used to elevate faster wound healing and commercially adopted in wound dressing, different pharmaceutical dosage formulation and medical implant coating. Other metal nanoparticles like platinum nanoparticles also evaluated for their health beneficial effect and successfully used in biomedical applications in either pure form or metal alloyed as a single or in combination with other metal nanoparticles. The use of metal nanoparticles is continuously increasing worldwide in biomedicine and allied disciplines. In view of this, present review is an assortment of various methods used for the preparation of metallic nanoparticles, their advantages, disadvantages and applications **(Metzel, 1990).**

Consequently, a number of research works have been published, reporting the initiatives done by researchers to provide nutritional supplements in many forms; one of them is through the development of NPs. Nano-sized particles are capable to work as pharmaceutical carriers for various delivery system; orally, transdermal, or intravenously. Studies show that NPs have been tremendously used in the biomedical sector to treat diseases like diabetes and cancer. Additionally, particle size plays an important role in controlling the efficiency of transporting therapeutic agents by conventional ways. NPs are favoured to deliver the medicine because their size is small and they have high surface area for better pharmaceutical release to the target organ. Stage alteration of the polymer and encapsulation are needed to keep on producing better therapeutic delivery systems. However, in innovating the transport system for therapeutic agents, many challenges need to be solved; one of them is the selection of nanomaterial. NPs can be made up of a list of materials but some existing NPs may be harmful to humans as they may generate toxicity to the body and environment during their production, application, and disposal. Some materials for NPs formation, mainly metal based are reported to cause undesirable side effects to living organisms **(Kumari et al., 2021).**

1.3 Silver Nanoparticles

Silver nanoparticles (AgNPs) are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties. These include optical, electrical, and thermal, high electrical conductivity, and biological properties. Due to their peculiar properties, they have been used for several applications, including as antibacterial agents, in industrial, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopaedics, drug delivery, as anticancer agents, and have ultimately enhanced the tumour-killing effects of anticancer drugs. Recently, AgNPs have been frequently used in many textiles, keyboards, wound dressings, and biomedical devices. Nanosized metallic particles are unique and can considerably change physical, chemical, and biological properties due to their surface-to-volume ratio; therefore, these nanoparticles have been exploited for various purposes. In order to fulfil the requirement of AgNPs, various methods have been adopted for synthesis. Generally, conventional physical and chemical methods seem to be very expensive and hazardous. Interestingly, biologically-prepared AgNPs show high yield, solubility, and high stability. Among several synthetic methods for AgNPs, biological methods seem to be simple, rapid, non-toxic, dependable, and green approaches that can produce well-defined size and morphology under optimized conditions for translational research. In the end, a green chemistry approach for the synthesis of AgNPs shows much promise. After synthesis, precise particle characterization is necessary, because the physicochemical properties of a particle could have a significant impact on their biological properties. In order to address the safety issue to use the full potential of any nano material in the purpose of human welfare, in nanomedicines, or in the health care industry, etc., it is necessary to characterize the prepared nanoparticles before application. The characteristic feature of nanomaterials, such as size, shape, size distribution, surface area, shape, solubility, aggregation, etc. need to be evaluated before assessing toxicity or biocompatibility **(Zhang et al., 2016).**

Nanoparticles can be synthesized chemically or biologically. Many adverse effects have been associated with chemical synthesis methods due to the presence of some toxic chemical absorbed on the surface. Eco friendly alternatives to Chemical and physical methods are biological ways of nanoparticles synthesis using microorganisms, enzymes, fungus and plants or plant extracts. The development of these eco-friendly methods for the synthesis of nanoparticles is evolving into an important branch of nanotechnology especially silver nanoparticles, which have many applications. Mechanism Biosynthesis of nanoparticles by microorganisms is a green and eco-friendly technology. Diverse microorganisms, both prokaryotes and eukaryotes are used for synthesis of metallic nanoparticles viz. silver, gold, platinum, zirconium, palladium, iron, cadmium and metal oxides such as titanium oxide, zinc oxide, etc. These microorganisms include bacteria, actinomycetes, fungi and algae. The synthesis of nanoparticles may be intracellular or extracellular according to the location of nanoparticles.

Objectives of the present work

- To synthesis the silver nanoparticles using Thymoquinone.
- To confirm the formation of bio conjugated AgNPs with TQ using spectroscopy techniques.
- To evaluate the antioxidant and antimicrobial activities of bioconjugated AgNPs with TQ.
- To identify the binding pocket of SARS-CoV-2 protease protein with the interaction of TQ using molecular docking studies.

CHAPTER – II

REVIEW OF LITERATURE

2.1 Introduction

This chapter deals with the literature review on the synthesis of metal nanoparticles by thymoquinone present in *Nigella sativa* seeds. Additionally, the biological activities of bioconjugation of AgNPs and interaction with protein by thymoquinone have also reviewed in this chapter.

2.2 Overview of Literature

Mohamed Mahmoud Fathy (2019) prepared biosynthesis of Silver Nanoparticles using Thymoquinone and evaluated their Radio-Sensitizing Activity. Radiation therapy deals with providing an optimum radiation dose to the target and to preserve the surrounding normal tissues. Silver nanoparticles (AgNPs) have been used for a wide range of biomedical applications based on their surface modifications, sizes and shapes. This study deals with green synthesized nano-formulations using Thymoquinone (TQ), which is used as reducing and capping agent with which assessment of their radio-sensitizing effect against the highly aggressive MDA-MB-231 mammary adenocarcinoma. Different sizes of AgNPs were prepared by changing the amounts of added TQ. Different physical techniques were used to characterize the prepared silver nano-formulations. Using the MTT cytotoxicity assay, the radio-sensitization effectiveness of produced AgNPs has been evaluated. Neutral comet test was used as a follow-up evaluation to look into how many DNA double-strand breaks resulted from AgNPs radio-sensitization. The average size of the generated silver nanoparticles (AgNPs-20, AgNPs-15, and AgNPs-10) was gradually reduced to be 20 1.5 nm, 15 2 nm, and 10 1.8 nm, respectively, as the amount of the added TQ rose from 10, 20, and 40 mg. DLS measurements and UV-Vis spectroscopy supported this. AgNPs had a size- and concentration-dependent radio-sensitization activity, according to the results of the Comet and MTT assays, however the radiation dose augmentation for small-sized nanoparticles (AgNPs-10) was higher than that for larger-sized nanoparticles (AgNPs-20 and AgNPs-15). Therefore, thymoquinone capping silver nanoparticles show good nano-formulation for enhancing cancer radiosensitivity.

Basma Salama et al., (2022) found that thymoquinone added silver nanoparticles play a protective role in enhancing oxidative stress, inflammation, and apoptosis in liver and kidney tissues. The purpose of this study was to determine whether thymoquinone (TQ) could protect rats against the cytotoxicity caused by AgNPs to the liver and kidneys. The condition of liver and kidney oxidative stress, pro-inflammatory cytokines, apoptotic indicators, and histopathology were all evaluated along with serum markers of liver and kidney functioning. Aspartate transaminase, alanine transaminase, urea, and creatinine were among the blood liver and kidney function markers that AgNPs elevated. TQ corrected these effects. Additionally, TQ co-administration with AgNPs reduces hepatic and renal oxidative insults by lowering MDA and NO levels with a significantly higher level of antioxidant enzyme activity (superoxide dismutase, catalase, and glutathione recycling enzymes peroxidase and reductase) compared to AgNPs-treated rats. Moreover, TQ co-administration reduced the levels of IL-1, TNF-, TGF-, and NF-B, which reflect hepatic and renal pro-inflammatory mediators. Moreover, TQ co-administration enhanced the levels of the anti-apoptotic protein (Bcl-2) and lowered the levels of the apoptotic protein. The histological analysis of hepatic and renal tissues supported these findings. Our findings supported TQ's ability to shield cells from the cytotoxicity of AgNPs and suggested a potential mechanism for its antioxidant, anti-inflammatory, and anti-apoptotic properties. As a result, we might draw the conclusion that utilising TQ might reduce the toxicological effects of AgNPs, raise their safety, and improve the effectiveness of their applications.

Amira Y. Mahfouz et al., (2021) reported the eco-friendly and superficial approach for synthesis of silver nanoparticles using Nigella sativa and Piper nigrum L aqueous extracts which was further used for evaluation of antibacterial, antiviral and anticancer activities which impacts its study on its impact on seed germination and seedling growth of Vicia faba and Zea mays. AgNPs were produced during this experiment by reducing AgNO3 with aqueous seed extracts of Nigella sativa and Piper nigrum. Transmission electron microscopy, Fourier-transform infrared spectroscopy, zeta potential, and dynamic light scattering were used to characterise the biosynthesized AgNPs. AgNPs antibacterial, antiviral, and anticancer properties were investigated. Also, it was evaluated what impact biosynthesized AgNPs had on the seed germination and seedling development of Zea mays and Vicia faba.

Rayhaneh Amooaghaie et al., (2015) studied synthesis, characterization and biocompatibility of silver nanoparticles synthesized from Nigella sativa leaf extract in comparison with chemical silver nanoparticles. In this study, "green" and "chemical" methods were used to create silver nanoparticles (AgNPs). Silver nitrate, sodium citrate, and sodium borohydrate were the raw materials employed in the wet-chemistry process. In the green synthesis process, silver nitrate was reduced using a leaf extract from the Nigella sativa plant as both a capping and reducing agent. Also, the toxicity of both produced AgNPs was observed in mouse bone-forming stem cells as well as in the germination and seedling development of six different plant species (Lolium, wheat, bean and common vetch, lettuce and canola). The colourless reaction mixtures turned brown in both synthesis techniques, and UV-visible spectra proved that silver nanoparticles were present. The prevalence of silver nanosized crystallites was revealed by scanning electron microscopy (SEM) measurements, and the significance of various functional groups in the synthetic process was disclosed by Fourier transform infrared spectroscopy (FTIR). The MTT assay revealed that the green AgNPs produced using black cumin extract were more effective in preserving mouse bone-forming stem cells than chemical AgNPs. For six plants exposed to green AgNPs, IC50 values for seed germination, root length, and shoot length were higher than those for chemical AgNPs. These findings imply that the cytotoxicity and phytotoxicity of AgNPs produced using green chemistry were much lower than those produced using wet chemistry. This study indicated an economical, simple, and efficient eco-friendly technique using leaves of N. sativa for synthesis of AgNPs and confirmed that green AgNPs are safer than chemically-synthesized AgNPs.

Maha I. Alkhalaf et al., (2020) reported the green synthesis of silver nanoparticles by Nigella sativa extracts alleviates diabetic neuropathy through anti-inflammatory and antioxidant effects. In this study, 50 adult male albino rats were used and they were divided into five groups. Groups (3-6) were diabetic neuropathy induced group and treated with Nigella sativa extract and green synthesized silver nanoparticles. Biochemical parameters such as diabetic, inflammatory, antioxidant biomarkers and brain histopathology were evaluated. The results showed a significant increase in glucose, AGE, and aldose reductase with insulin reduction in the diabetic neuropathy induced group compared to the healthy control group. In addition, inflammatory markers increased significantly in the diabetic neuropathy group. The same group experienced a remarkable change in oxidative status. Furthermore, nitrotyrosin levels decreased significantly. In terms of gene expression, we discovered a significant decrease in brain TKr A and an increase in nerve growth factor in the diabetic neuropathy group compared to the healthy control group. Several treatments for diabetic neuropathy significantly improve all of the biomarkers studied. The results of this study are heavily reliant on histological findings. Therefore, it can be established that green synthesis of silver nanoparticles in combination with Nigella sativa extract could be a newly neuroprotective agents against inflammation and oxidative stress characterizing diabetic neuropathy through their antidiabetic, anti-inflammatory and anti-oxidants effects.

Nancy Jain et al., (2021) prepared green synthesized plant-based silver nanoparticles which was used as therapeutic prospective for anticancer and antiviral activity. Nanotechnology is an emerging field of medical science because it can be used in almost any situation. Phyto-constituents are promising candidates for synthesising green silver nanoparticles (AgNPs), which have great potential for treating chronic diseases. This review provides an overview of the green approach to the synthesis and characterization of AgNPs. The current review delves deeper into the potentials of Phyto-based AgNPs for anticancer and antiviral activity, as well as the likely mechanism of action. Green synthesised AgNPs prepared from a variety of medicinal plant extracts are being tested for cancer and viral infection. Thus, in a single window, this article focuses on green synthesised Phyto-based AgNPs and their potential applications for cancer and viral infection, including mechanism of action and therapeutic future prospects.

Zainab Sattar Ali et al., (2022) reported synthesis, characterization of silver nanoparticles using Nigella sativa seeds and study their effects on the serum lipid profile and DNA damage on the rat's blood treated with hydrogen peroxide. The goal of this study was to create silver nanoparticles from aqueous extract of Nigella sativa, as well as to investigate the effects of green synthesised Nigella sativa seeds silver nanoparticles on dyslipidaemia and DNA fragmentation in rats exposed to hydrogen peroxide. The silver nanoparticles synthesised from Nigella sativa seeds were characterised using ultraviolet-visible spectroscopy, Fourier-transform infrared spectroscopy, X-ray powder diffraction (XRD) style, and a scanning electron microscope to investigate the morphology and size. In conclusion, the current study results show that Nigella sativa seeds silver nanoparticles have an ameliorative effect on lipid profile and DNA damage.

Ezzat H. Elshazly et al., (2022) reported phytotoxicity and antimicrobial activity of green synthesized silver nanoparticles using Nigella sativa seeds on Wheat seedlings. Aqueous extracts of Nigella sativa (N. sativa) seeds were used to create silver nanoparticles (AgNPs). An X-ray diffractometer, a UV-visible spectrometer, and a transmission electron microscope were used to confirm the formation of AgNPs. Wheat (Triticum aestivum L.) seed germination was used to assess the phytotoxicity and genotoxicity of various AgNP concentrations (12.5, 25, 50, 75, and 100 gL1). The findings revealed that AgNPs had no effect on germination, but significantly reduced root and coleoptile lengths. On the contrary, seedling biomass increased significantly in response to AgNP treatments. Furthermore, genotoxicity was discovered, particularly at high AgNP concentrations. Wheat seedlings' DNA, RNA, and total soluble proteins all decreased significantly. Furthermore, antimicrobial activities of biosynthesized AgNPs were discovered.

Marvit Osman Widdatallah et al., (2020) reported green synthesis of silver nanoparticles using Nigella sativa seeds and evaluated their antibacterial activity. This study looked at the synthesis of silver nanoparticles using Nigella sativa seeds as a capping agent. Sunlight was used to accelerate the formulation of different concentrations of N. sativa aqueous extract with silver nitrate solution. UV-Vis, scanning electron microscope (SEM), and X-ray diffraction (XRD) techniques were then used to characterize the silver nanoparticles. The disc diffusion method was used to test the antibacterial activity of nanoparticles against Staphylococcus aureus and Escherichia coli. The characterization of nanoparticles was detected by a color change to yellow-brown, indicating the formation of silver nanoparticles. SEM and XRD techniques were used to detect irregular shapes on the nanoscale. The discovery suggests that silver nanoparticles could be used effectively as an antibacterial agent.

Anitha. S et al., (2022) UV-Vis absorption and fluorescence spectroscopy were used to investigate the interaction of two stereoisomeric flavanols (+) catechin (CT) and (+) epicatechin (ECT) with bovine serum albumin (BSA). Fluorescence research revealed that both flavanols interact strongly with BSA at a single binding site. Molecular docking studies were used to identify the binding pocket of BSA at site I/II with the interaction of CT/ECT. Molecular dynamics simulation (MDS) revealed that the interaction of CT/ECT caused structural alterations in BSA, resulting in strong -helix forms. The MDS result indicates that CT/ECT binding to BSA is due to hydrophobic and hydrogen bond interactions, which supports the experimental findings. CT/ECT have different binding sites and structural effects due to different orientational positions at the benzopyran moiety.

Tony Fröhlich et al., (2017) reported a series of hybrid compounds based on the natural products artemisinin and thymoquinone was synthesized and investigated for their biological activity against the malaria parasite Plasmodium falciparum 3D7 strain, human cytomegalovirus (HCMV) and two leukemia cell lines (drug-sensitive CCRF-CEM and multidrug-resistant sub-line CEM/ADR5000). An unprecedented one-pot method of selective formation of C-10α-acetate 14 starting from a 1:1 mixture of C-10α- to C-10β-dihydroartemisinin (DHA), was developed. The key step of this facile method is a mild decarboxylative activation of malonic acid mediated by DCC/DMAP.Moreover, they showed to be 5 times more active than the standard drug ganciclovir and nearly 8 times more active thanartesunic acid against HCMV. In addition, hybrids **6a/b** possessed excellent antimalarial activity (EC50 =5.9/3.7 nm.), which was better than that of artesunic acid (EC50 = 8.2 nm.) and chloroquine (EC50 = 9.8 nm.). Overall, most of the presented thymoquinone artemisinin-based hybrids exhibit an excellent and broad variety of biological activities (anticancer, antimalarial, antiviral) combined with a low toxicity/high selectivity profile.

Siti Mariyah Ulfa et al., (2017) Thymoquinone derivatives which synthesized in this research is bromoalkylquinones with alkyl chain consist of seven carbons (C7) and ten carbons (C10). The synthesis was carried out by oxidation of 2,3-dimethylhydroquinone followed by alkylation using reflux for 1.5 hours. The alkylation products were successfully characterized as 5-(7-bromoheptyl)-2,3-dimethyl-1,4-benzoquinone (C7) and 5-(10-bromodecyl)-2,3-dimethyl-1,4-benzoquinone (C10) in 31.93 and 16.89%, respectively. These compounds were fully characterized using FT-IR, 1H-NMR and 13C-NMR. Thus, the activity of C7 and C10 was analysed by in silico approach with molecular docking using macromolecule model extracted from Protein Data Bank (PDB). Macromolecules used in this research is mitochondrial translocator protein (TSPO) as an antioxidant receptor, glycogen phosphorylase (GPA) as antidiabetic receptor and phosphatase tensin homolog (PTEN) as an anticancer agent. The result showed that C7 and C10 has a very good activity as antioxidant and antidiabetic agents with IC50 2.03 and 1.02 ppm (TSPO) and 16.98 and 14.88 ppm (GPA) compared with Thymoquinone. While the activity of C7 and C10 against PTEN gave the IC50 23.13 and 18.31 ppm showed a good candidate for an anticancer agent.

Osama A Badary et al., (2021) COVID-19 has caused a major global health crisis, as excessive inflammation, oxidation, and exaggerated immune response in some sufferers can lead to a condition known as cytokine storm, which may progress to acute respiratory distress syndrome (ARDs), which can be fatal. So far, few effective drugs have emerged to assist in the treatment of patients with COVID-19, though some herbal medicine candidates may assist in the fight against COVID-19 deaths. Thymoquinone (TQ), the main active ingredient of black seed oil, possesses antioxidant, anti-inflammatory, antiviral, antimicrobial, immunomodulatory and anticoagulant activities. TQ also increases the activity and number of cytokine suppressors, lymphocytes, natural killer cells, and macrophages, and it has demonstrated antiviral potential against a number of viruses, including murine cytomegalovirus, Epstein-Barr virus, hepatitis C virus, human immunodeficiency virus, and other coronaviruses. Recently, TQ has demonstrated notable antiviral activity against a

SARSCoV-2 strain isolated from Egyptian patients and, interestingly, molecular docking studies have also shown that TQ could potentially inhibit COVID-19 development through binding to the receptor-binding domain on the spike and envelope proteins of SARS-CoV-2, which may hinder virus entry into the host cell and inhibit its ion channel and pore forming activity. Other studies have shown that TQ may have an inhibitory effect on SARS CoV2 proteases, which could diminish viral replication, and it has also demonstrated good antagonism to angiotensin-converting enzyme 2 receptors, allowing it to interfere with virus uptake into the host cell. Several studies have also noted its potential protective capability against numerous chronic diseases and conditions, including diabetes, hypertension, dyslipidemia, asthma, renal dysfunction and malignancy. TQ has recently been tested in clinical trials for the treatment of several different diseases, and this review thus aims to highlight the potential therapeutic effects of TQ in the context of the COVID-19 pandemic.
Keywords: thymoquinone, COVID-19, natural, therapeutic benefits.

CHAPTER - III
METHODOLOGY

3.1 Introduction:

The methods associated with producing silver nanoparticles and the formation of bioconjugates using TQ are thoroughly described in this chapter. This chapter also covers the methodology used to measure biological activities including antioxidant and antibacterial activity. The characterization techniques namely Ultraviolet-Visible Spectroscopy (UV-Vis), Fourier-Transform Infrared Spectroscopy (FTIR) and Transmission Electron Microscope (TEM) are discussed to study the formation of bioconjugates. The present study also focuses the computational details of plasma protein binding interaction of SARS-CoV-2 protease protein with TQ using docking methods.

3.2 Materials and Methods:

3.2.1 Chemicals Used:

Table 3.1 – List of chemicals used in the Experimental work

S. No	Chemicals used	Chemical formula	Make
1.	Thymoquinone	$C_{15}H_{14}O_{16}$	Sigma Aldrich
2.	Silver Nitrate	$AgNO_3$	Hi - media
3.	2,2-diphenyl-l-picrylhydrazyl (DPPH)	$C_{18}H_{12}N_5O_6$	Sigma Aldrich
4.	Muller-Hinton Agar		Hi media
5.	Methanol	CH_3OH	Nice
6.	Distilled Water		Aqua Gold

3.2.2 Synthesis of Silver nanoparticles:

Silver nanoparticles were prepared by silver nitrate reduction using TQ molecules as a reducing and capping agent. Briefly, 5ml of silver nitrate aqueous solution 0.6mM was left under stirring. Then 0.6mM of Thymoquinone was dissolved in 5ml of Methanol is been added to the silver nitrate aqueous solution dropwise at 60ºC. The solution was kept under stirring until the complete synthesis of AgNPs. The color change from a yellow (TQ color) to a light brown color indicated the successful preparation of silver nanoparticle capping with TQ. Figure 3.1 shows the color change due to the formation of AgNPs. The synthesized AgNPs were characterized by UV-Vis absorbance spectroscopy, FTIR, Raman Spectroscopy and TEM.

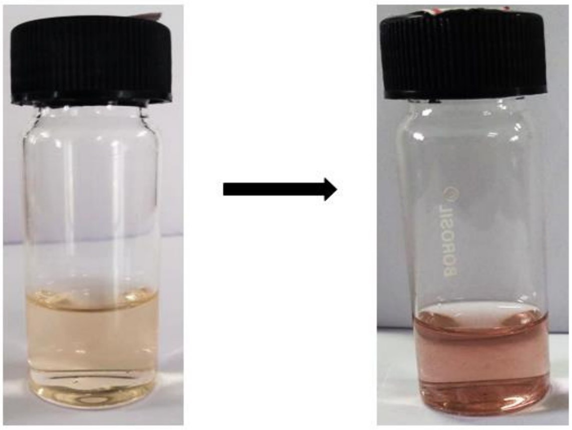

Figure 3.1: Synthesis of AgNPs-TQ

3.3 Characterization Techniques:

3.3.1 Optical Analysis

3.3.1.1 Ultraviolet-Visible (UV-Vis) Spectroscopy:

UV-Vis spectroscopy is an analytical technique that measures the number of discrete wavelengths of UV or visible light that are absorbed by or transmitted through a sample in comparison to a reference or blank sample. This property is influenced by the sample composition, potentially providing information on what is in the sample and at what concentration. To ascertain either AgNPs are developed or not visual and calorimetric appearance of samples checked by UV–Visible spectrophotometer before and after formulation of AgNPs at different time intervals. Electronic absorption spectra were recorded using UV–Visible absorption spectrophotometer (V-670-Jasco) in the range of 200–800 nm. Distilled water was used as blank solution.

Figure 3.2: Ultraviolet – Visible Spectrophotometer

Sample Analysis:

Whichever wavelength selector is used in the spectrophotometer, the light then passes through a sample. For all analyses, measuring a reference sample, often referred to as the "blank sample", such as a cuvette filled with a similar solvent used to prepare the sample, is imperative. If an aqueous buffered solution containing the sample is used for measurements, then the aqueous buffered solution without the substance of interest is used as the reference. When examining bacterial cultures, the sterile culture media would be used as the reference. The reference sample signal is then later used automatically by the instrument to help obtain the true absorbance values of the analytics. UV-Vis spectroscopy information may be presented as a graph of absorbance, optical density or transmittance as a function of wavelength. However, the information is more often presented as a graph of absorbance on the vertical y axis and wavelength on the horizontal x axis. This graph is typically referred to as an absorption spectrum **(Yadav, 2005).**

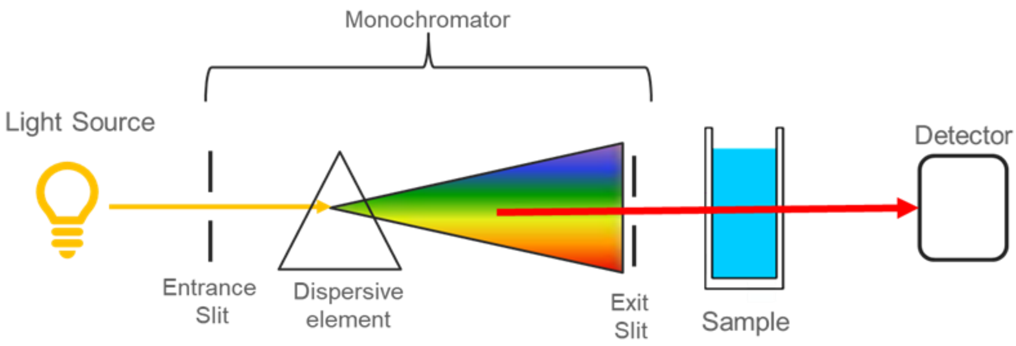

Figure 3.3: A simple schematic representation of the main components in UV-Vis Spectrophotometer

3.3.2 Morphological Analysis

3.3.2.1 TEM Analysis

The Transmission Electron Microscope (TEM) operates on many of the same optical principles as the light microscope. The TEM has the added advantage of greater resolution. This increased resolution allows us to study ultrastructure of organelles, viruses and macromolecules. Specially prepared materials samples may also be viewed in the TEM. The light microscope and TEM are commonly used in conjunction with each other to complement a research project TEM, type of electron microscope that has three essential systems:

(1) an electron gun, which produces the electron beam, and the condenser system, which focuses the beam onto the object,

(2) the image-producing system, consisting of the objective lens, movable specimen stage, and intermediate and projector lenses, which focus the electrons passing through the specimen to form a real, highly magnified image, and

(3) the image-recording system, which converts the electron image into some form perceptible to the human eye. The image-recording system usually consists of a fluorescent screen for viewing and focusing the image and a digital camera for permanent records. In addition, a vacuum system, consisting of pumps and their associated gauges and valves, and power supplies are required.

Figure 3.4: Transmission Electron Microscope

Working:

- A heated tungsten filament in the electron gun produces electrons that get focus on the specimen by the condenser lenses.
- Magnetic lenses are used to focus the beam of electrons of the specimen. By the assistance offered by the column tube of the condenser lens into the vacuum creating a clear image without collision with any air molecules which may deflect them.
- On reaching the specimen, the specimen scatters the electrons focusing them on the magnetic lenses forming a large clear image and if it passes through a fluorescent screen, it forms a polychromatic image.
- The denser the specimen, the more the electrons are scattered forming a darker image because fewer electron reaches the screen for visualization while thinner, more transparent specimens appear brighter.

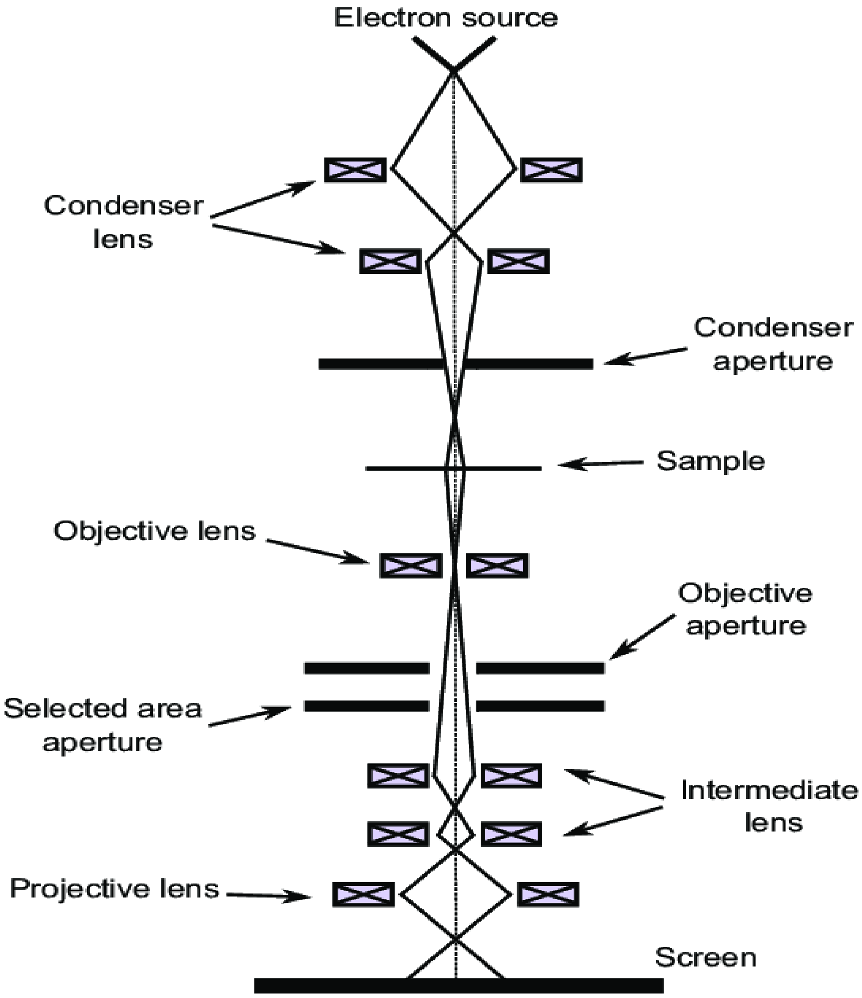

Figure 3.5: Schematic representation of components in Transmission Electron Microscope

Image Producing System:

- It is made up of the objective lens which is a movable stage or holding the specimen, intermediate and projector lenses. They function by focusing the passing electrons through the specimen forming a highly magnified image.
- The objective has a short focal length of about 1-5mm and it produces an intermediate image from the condenser which are transmitted to the projector lenses for magnification.
- The projector lenses are of two types, i.e., the intermediate lens which allows great magnification of the image and the projector lens which gives a generally greater magnification over the intermediate lens.
- To produce efficient high standard images, the objectives and the projector lenses need high power supplies with high stability for the highest standard of resolution.

Image Recording System:

- It is made up of the fluorescent screen used to view and to focus on the image. They also have a digital camera that permanently records the images captured after viewing.
- They have a vacuum system that prevents the bombardment or collision of electrons with air molecules disrupting their movement and ability to focus. A vacuumed system facilitates the straight movement of electrons to the image.
- The vacuumed system is made up of a pump, gauge, valves and a power supply.
- The image that is formed is called a monochromatic image, which is greyish or black and white. The image must be visible to the human eye, and therefore, the electrons are allowed to pass through a fluorescent screen fixed at the base of the microscope.
- The image can also be captured digitally and displayed on a computer and stored in a JPEG or TIFF format. During the storage, the image can be manipulated from its monochromatic state to a coloured image depending on the recording apparatus e.g., use of pixel cameras can store the image in colour.
- The presence of coloured images allows easy visualization, identification, and characterization of the images.

Applications of Transmission Electron Microscope (TEM)

TEM is used in various fields such as Biology, Microbiology, Nanotechnology, Forensic studies etc. Some of the applications include:

- To visualize and study cell structures of bacteria, viruses and fungi.
- To view bacteria flagella and plasmids.
- To view the shapes and sizes of microbial cell organelles.
- To study and differentiate between plant and animal cells.
- It is also used in nanotechnology to study nanoparticles such as ZnO nanoparticles.
- It is used to detect and identify fracture, damaged microparticles which further enable repair mechanisms of the particles **(Kannan, 2018).**

3.3.3 FTIR Spectroscopy:

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas.

Figure 3.6: Fourier Transform Infrared Spectrometer

A spectrometer is an optical instrument used to measure properties of light over a specific portion of the electromagnetic spectrum, 5 microns – 20 microns. FTIR (Fourier Transform InfraRed) spectrometer obtains an infrared spectrum by first collecting an interferogram of a sample signal using an interferometer, then performs a Fourier Transform on the interferogram to obtain the spectrum. An interferometer is an instrument that uses the technique of superimposing two or more waves to detect differences between them. The FTIR spectrometer uses a Michelson interferometer **(Griffiths et al., 2006).**

Applications of FTIR Spectroscopy:

- Identification of inorganic compounds and organic compounds.
- Identification of components of an unknown mixture.
- Analysis of solids, liquids and gases.
- In remote sensing.
- In measurement and analysis of Atmospheric spectra
    - Solar irradiance at any point of earth
    - Longwave / terrestrial radiation spectra
- Can also be used on satellites to probe the space.

3.4 Investigation of Biological Activity:

3.4.1 2, 2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging activity

The free radical scavenging efficacy of the bioconjugation of silver nanoparticles was determined using DPPH assay. Different concentrations of TQ and TQ-AgNPs ranging from 5 to 50 µL were added to 500 µL of DPPH (0.3 mM of DPPH). The solutions were mixed thoroughly and incubated in the dark for 30min at room temperature. Ascorbic acid is used as the standard reference to compare the antioxidant activity of all above samples. The percentage of DPPH radical scavenging ability was calculated to test the antioxidant activity of all the samples using the following equation.

$$\text{Percentage of radical scavenging ability} = \left[\frac{Acontrol - Asample}{Acontrol}\right] X\ 100 \qquad (1)$$

Where, A_{sample} is the absorption of DPPH radical solution with TQ and TQ-AgNPs and $A_{control}$ is the absorbance of pure DPPH radical solution **(Anitha et al., 2022).**

3.4.2 Antibacterial Activity

The antibacterial activity of TQ and bioconjugation of AgNPs with TQ is performed by Agar well diffusion method using Muller-Hinton Agar as growth media. The bacterial strains namely Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative) are used in this study. Sterile agar plates were inoculated and 30 µL of TQ and bioconjugate TQ-AgNPs were added separately into the wells bored in the agar medium. Then the plates were incubated at 37^{o}C for 24 hrs. A well loaded with antibiotic, ampicillin served as positive control and was maintained on each plate.

3.5 Molecular Docking

Computational docking is a method used to predict the binding mode and affinity of a ligand molecule to a target protein. It involves using computational algorithms and software to simulate the interaction between the two molecules, and to predict the conformation of the ligand molecule that is most likely to bind to the protein with high affinity. Single docking experiments are useful for exploring the function of the target, and virtual screening, where a large library of compounds is docked and ranked, may be used to identify new inhibitors for drug development. Auto Dock is a suite of free open–source software for the computational docking and virtual screening of small molecules to macromolecular receptors. Auto Dock/Vina was employed for docking using protein and ligand information along with grid box properties in the configuration file. The pose with lowest energy of binding or binding affinity was extracted and aligned with receptor structure for further analysis. The SARS-CoV-2 protease protein structure (PDB ID: 6LU7) was taken from Protein Data Bank. The TQ ligand structure was taken from PubChem. Docking approach has been effectively used by research team against 6LU7 therapeutic targets. The Molecular Docking was applied to TQ and its analogues against the SARS-CoV-2 **(Anitha et al., 2022).**

CHAPTER – IV

RESULTS AND DISCUSSION

4.1. Introduction:

This study focuses on understanding the interaction of biosynthesized silver nanoparticle (AgNPs) with TQ. The binding characteristics of the TQ-AgNPs were explored by UV-Vis Spectroscopy. FTIR is used to analyse and identify the major functional groups responsible for formation of TQ-AgNPs. The size of biosynthesized TQ-AgNPs was confirmed by Transmission Electron Spectroscopy (TEM). The binding pockets of Covid protein with TQ were identified using molecular docking methods.

4.2 Analysis of formation of Silver Nanoparticle:

4.2.1. UV-Vis Spectral analysis of silver nanoparticles:

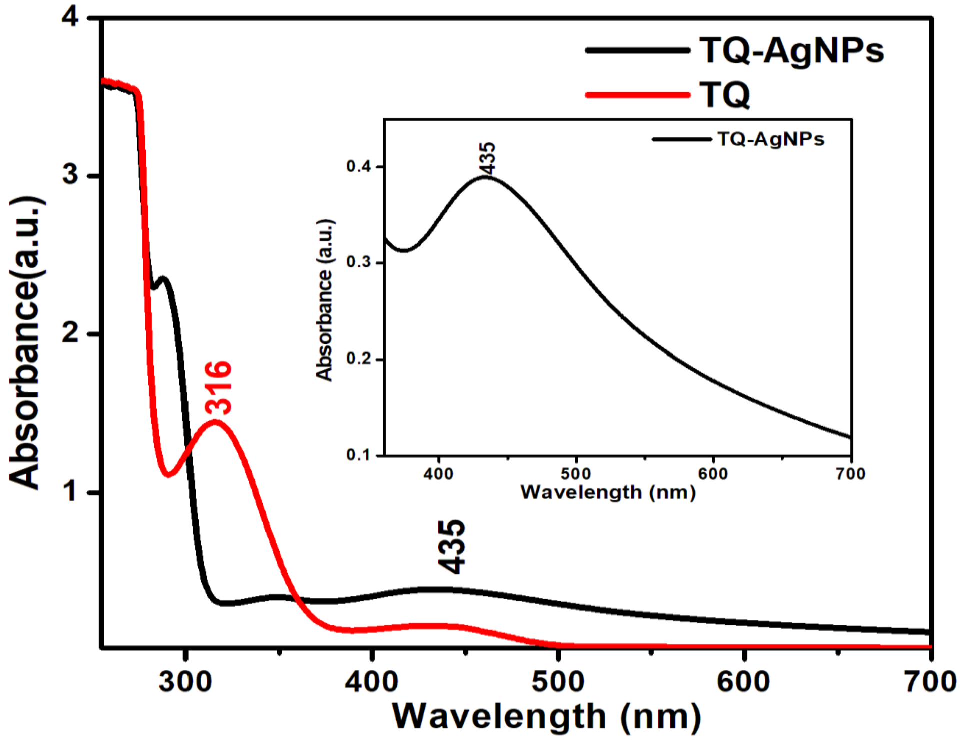

Figure 4.1 UV-Vis absorption spectra of TQ-AgNPs and TQ

The formation of synthesized nanoparticles is confirmed using UV-Vis Absorption spectral analysis. The UV-Vis absorption spectra of TQ-AgNPs and TQ are presented in Figure 4.1. UV-Vis absorption spectra of TQ shows peak at 316 nm. On addition of TQ to the $AgNO_3$ solution, the yellow colour solution changes to light brown colour, indicates the formation of AgNPs in the mixture. The highest absorbance broad peak at the wavelength of 435 nm, which shows the surface plasma resonance of the synthesized nanoparticles. This suggests the formation of unique spherical shaped nanoparticles. It confirmed that the compound TQ was firmly attached to the surface of synthesized $AgNO_3$ nanoparticles **(Anitha et al., 2022).**

4.2.2. Fourier Transform Infrared Spectroscopy

FTIR is used to obtain information about the functional groups involved in bioconjugation of AgNPs with TQ. The FTIR Spectra of TQ-AgNPs and TQ is shown in Figure. 4.2. The TQ spectrum has peak at 2967cm^{-1} region, which is attributable to stretching vibrations of the isopropyl and CH_3 groups. This peak is shifted to 3321 cm^{-1} with higher intensity for TQ-AgNPs. This result showed that the isopropyl groups of TQ involved in the bioconjugation of silver nanoparticles. The bending vibration of C-H bonds of TQ appeared at 672 cm^{-1} had been shifted to 710 cm^{-1} for TQ-AgNPs, this result indicate that the C-H groups are involved in the synthesis of silver nanoparticles by TQ **(Prathiba Gnanasekaran et al., 2021)**

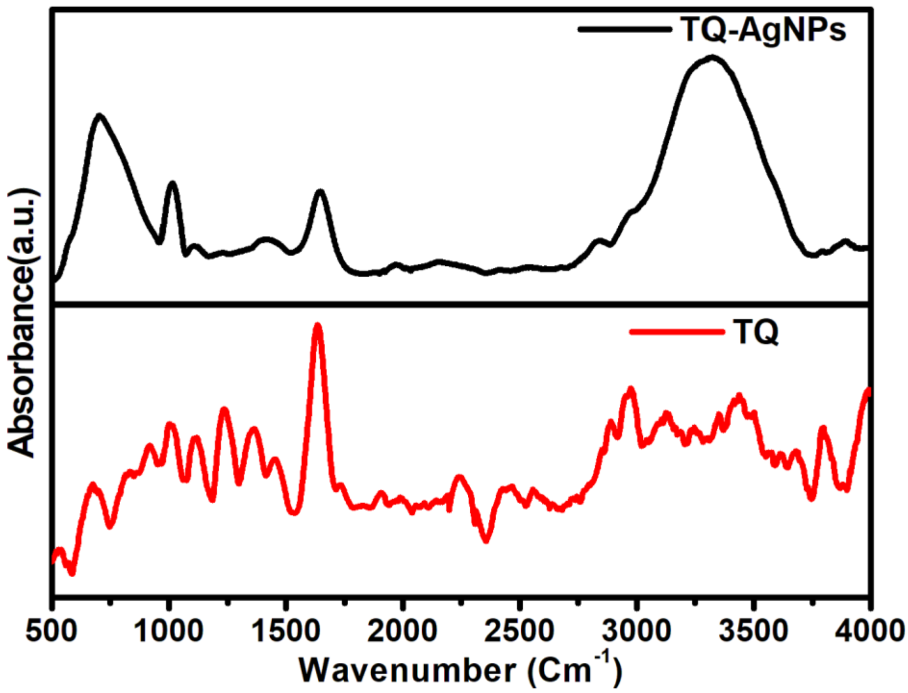

Figure. 4.2. FTIR spectra of TQ-AgNPs and TQ

4.2.3. Transmission Electron Microscopy

Transmission electron microscopy was used to investigate the morphology and size of synthesised silver nanoparticles. Fig. 3 (a-b) shows TEM images of synthesized silver nanoparticles using TQ. Synthesized nanoparticles have spherical shape and their size ranges from 35-38nm. The selected area of electron diffraction pattern indicated that the AgNPs are crystalline in nature, and the bright circular rings signify the presence of different AgNPs planes as shown in Fig.3 (c) **(Anitha et al., 2022).**

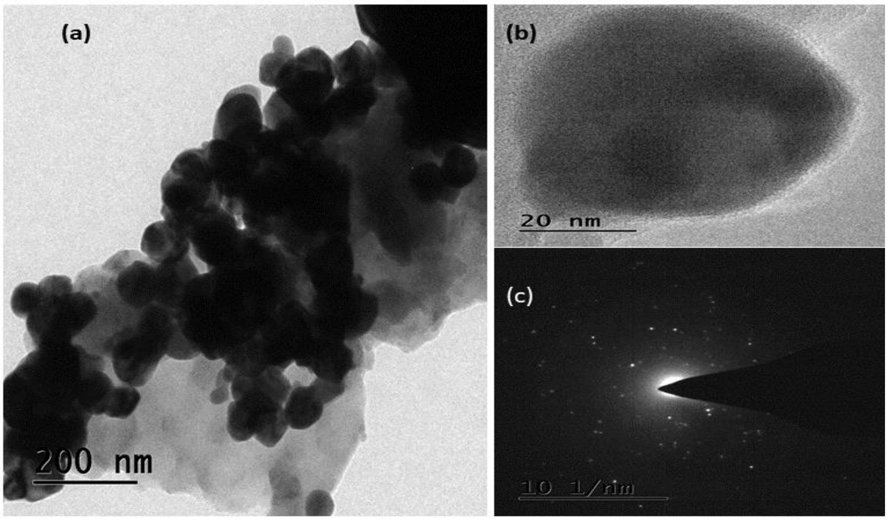

Figure 4.3: TEM images of (a) and (b) Bioconjugation of bioderived silver nanoparticles with TQ (AgNPs-TQ) (c) SAED pattern of AgNPs-TQ

4.3Antioxidant activity against DPPH radical

DPPH assay was implemented to investigate the antioxidant ability of bio and chemically conjugated silver nanoparticles. At room temperature, DPPH is a stable free radical and either by exchanging an electron or abstracting a hydrogen atom from antioxidants becomes a stable molecule **(Anitha et al., 2021)**. The scavenging activity of pure TQ and TQ-AgNPs are estimated with the concentration of 5-50 µL/mL against DPPH. The maximum optical absorbance at 517 nm gets decreases while adding the increasing concentration of pure antioxidants and bioconjugates. The percentages of scavenging abilities are estimated using equations and are given in Table 4.1. From the linear plot percentage of inhibition vs. the sample concentration shown in Figure 4.4. The half maximal inhibitory concentration (IC_{50}) values are calculated. It is found to be 32.03 and 24.50 µL/mL for TQ and TQ-AgNPs respectively. The observed free-radical-scavenging potential of entire bio conjugated AgNPs had lower IC_{50} values as compared to the pure extract of TQ **(Anitha et al., 2022).**

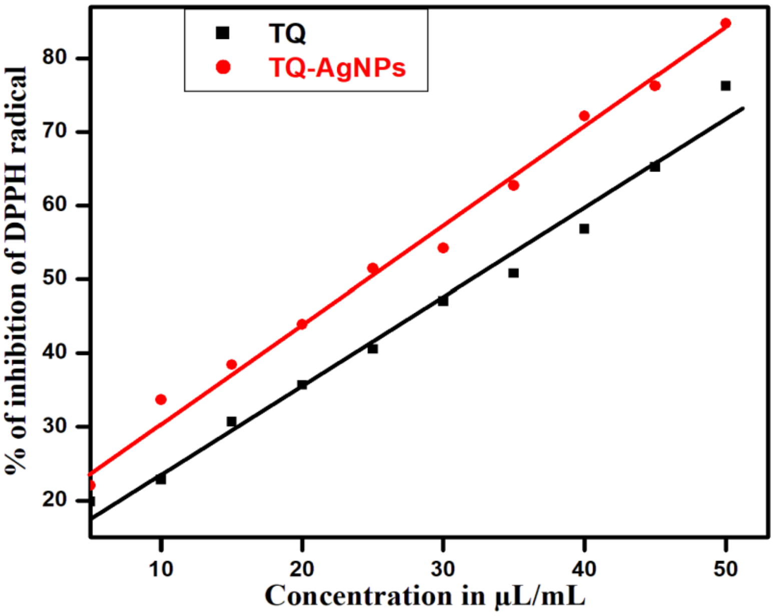

Figure 4.4: Percentage of inhibition of DPPH radical with various concentrations of TQ and TQ-AgNPs.

Table 4.1: Percentage of scavenging with respect to concentration of TQ and TQ-AgNPs against DPPH radical

Concentration µL/mL	Scavenging ability of TQ in %	IC 50 value in µL/mL	Scavenging ability of TQ-AgNPs in %	IC_{50} value in µL/mL
5	19.83	32.03	22.05	24.50
10	22.86		33.69	
15	30.71		38.44	
20	35.68		43.92	
25	40.58		51.49	
30	47.07		54.23	
35	50.78		62.69	
40	56.82		72.13	
45	65.21		76.26	
50	76.25		84.74	

4.4Antibacterial activity:

The antibacterial activities are estimated by well diffusion method against *Escherichia coli (E. coli)* (Gram-negative bacteria) and *Staphylococcus aureus (S. aureus)* (Gram-positive bacteria) for TQ and AgNPs-TQ as shown in Figure 4.5. The release of Ag+ ions from AgNPs to bacterial cells is responsible for the improved bactericidal impact of AgNPs. The diameter of the inhibition zones is measured. TQ, AgNPs-TQ and Control showed 23mm, 25mm and 15mm zone of inhibition against *S. aureus* and TQ, AgNPs-TQ and Control showed 21mm, 33mm and 19mm zone of inhibition against *E. coli*. The maximum zone of inhibition of 25mm was found against E. coli **(Anitha et al., 2022).**

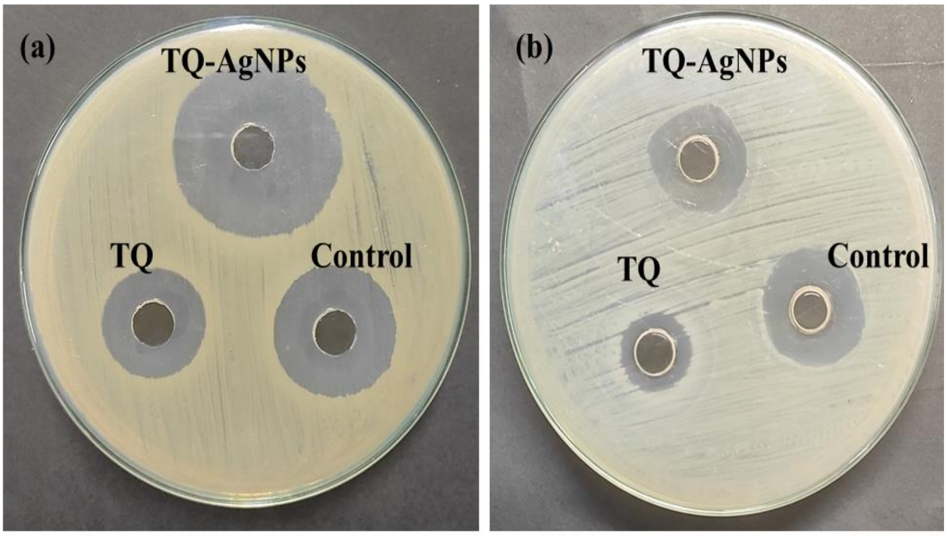

Figure 4.5: Antibacterial activity with zone of inhibition of TQ and TQ-AgNPs against (a) *Escherichia coli (E. coli)* and (b) *Staphylococcus aureus (S. aureus)*

4.5 Molecular Docking

Molecular Docking is a method that may be used to screen various compounds for their antiviral potential by testing the binding affinities of the compounds against different viral or host cell receptor proteins (**Badary et al., 2021**). The binding affinity of CoV-TQ has been found to be -5.2 kcal/mol. The docking studies were used to rank the compounds according to their binding affinity (kcal/mol) as shown in Table 4.2. The molecular docking conformations of CoV-TQ with lowest binding energy are presented in Figure 4.6. During the interaction, the amino acid residues surrounding TQ were HIS172, HIS163, PHE140, LEU141, ASN142, HIS41, MET165 respectively.

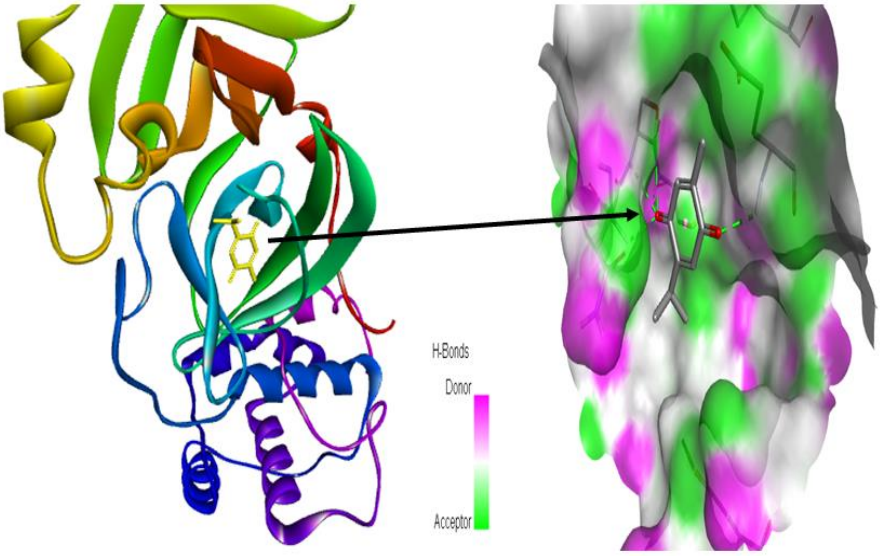

Figure 4.6: The molecular docking confirmation of CoV-TQ complex with high binding affinity

The binding of TQ with CoV protein occurs by hydrogen bond interaction through hydroxyl groups of TQ and polar residues of CoV, as well as hydrophobic interaction between TQ planar ring surfaces and non-polar residues of CoV. Figure 4.7 shows the possible hydrophobic interaction between residues of CoV and TQ. The red arcs labelled with hydrophobic residues of CoV encircled by TQ are MET165, HIS163, LEU141, CYS145, ASN142 and HIS41 respectively.

Table 4.2: Binding Affinity involved in docking interactions

Mode	Binding Affinity (kcal/mol)
1	-5.2
2	-5.1
3	-5.1
4	-5.0
5	-4.9
6	-4.8

7	-4.7
8	-4.7
9	-4.6

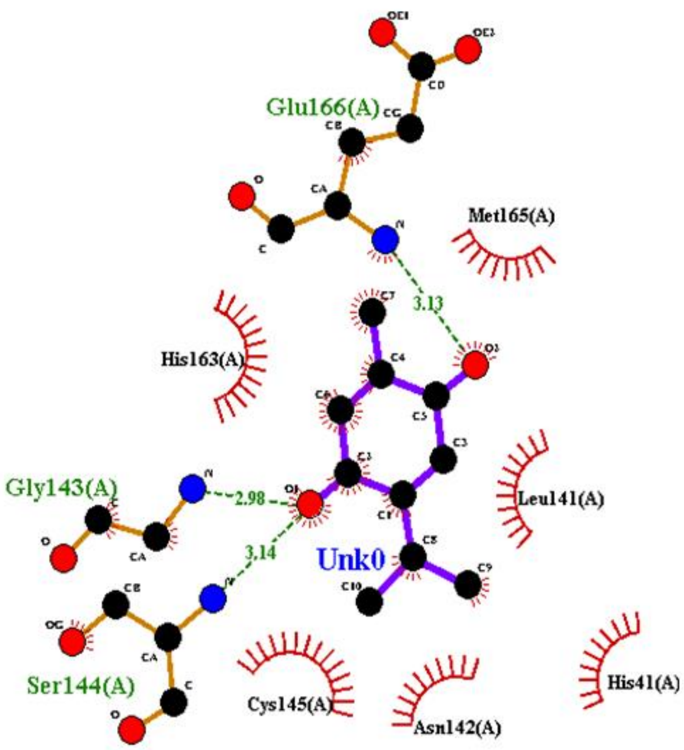

Figure. 4. 7. Two dimensional representations of ligplot of hydrophobic interaction residues of CoV with TQ.

The structural stability of the biomolecular complexes is sustained by the formation of hydrogen bond. In the case of the CoV- TQ complex, four hydrogen bonds are formed between the TQ and the amino acid residues (Figure. 4.8) are CYS145, GLY143, SER144 and GLU166 respectively. The above result showed the hydrophobic and hydrogen bond interactions are the major factors to stabile the CoV protein after interaction with TQ **(Anitha et al., 2022).**

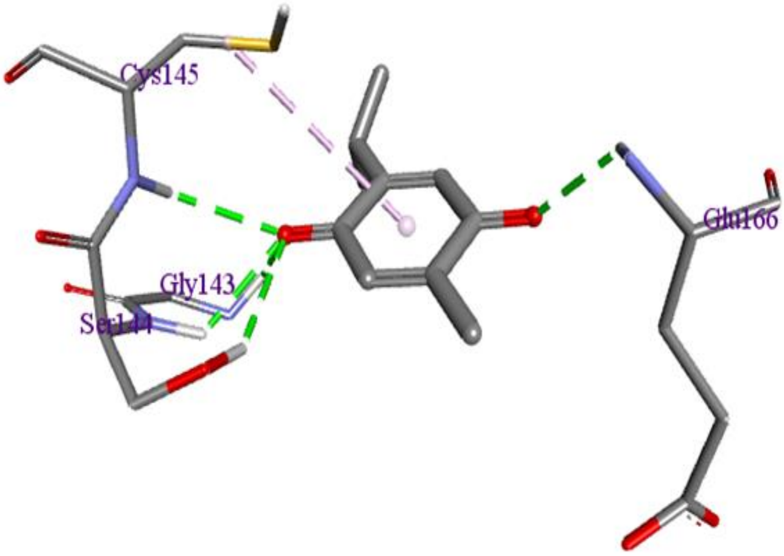

Figure. 4.8. Three dimensional conformations for amino acids surrounding TQ

CHAPTER – V

SUMMARY AND CONCLUSION

In the present study, the silver nanoparticle was biosynthesized using Thymoquinone. The formation of AgNPs was observed by the colour change from yellow colour to light brown confirms the formation of AgNPs. The obtained absorption maximum at 435 nm indicates the formation of AgNPs due to the bio reduction of Ag^+ to Ag^0 from the hydroxyl groups of TQ. FTIR spectra confirmed that the CH_3, isopropyl and C-H groups of TQ are involved in the bioconjugation of silver nanoparticles. TEM studies revealed that the spherical shaped nanoparticles were in the range of 35-38 nm. The bioconjugation of AgNPs with TQ showed the strongest synergistic antioxidant activity against 2, 2-diphenyl-1 picrylhydrazyl (DPPH) and antibacterial activity against *S. aureus and E. coli*. Molecular docking study confirmed that the hydrophobic and hydrogen bonding interactions are the major factors to stabilize the CoV protein after interaction with TQ.

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