Title: Synthesis and Characterization of Iron oxide Nanoparticles with Thymoquinone.

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Abstract

*Nigella sativa* is a medicinal plant used for its antimicrobial properties. Thymoquinone (TQ) being the lead component of this plant seeds, exerts antibacterial, antifungal and antioxidant activity. It has also shown promising activity against cancer and inflammation through different modes of action.

In recent developments for new applications in the field of medicine, nanotechnology has excelled as a very prominent and important field. Nanoparticles are generally in the dimension range of 1–100 nm. Iron oxide nanoparticles show enhanced antibacterial activity against gram-negative bacteria. The current research focuses on comparing the yield, stability and purity of thymoquinone synthesised iron oxide nanoparticles using different organic solvents, the benefits expressed by thymoquinone as a herbal drug and a novel approach to green synthesis of iron oxide nanoparticles with thymoquinone and testing for their stability, size and functional groups.

Keywords: Iron Oxide nanoparticles; *Nigella sativa*; Thymoquinone (TQ); Preparatory High-Pressure liquid chromatography; FTIR analysis; Zeta potential; Dynamic light scattering

Introduction

Currently, we are in the era of “End to Modern Medicine if bacteria can resist” *(Yoshikawa, 2002)*
It is important to accept that a majority of infections caused by bacteria are resistant to antibiotics thereby making them ineffective. An experiment was conducted by Stuart B Levy (2005) where three geographically separated patients treated with the antibiotic Vancomycin were subjected to the harmful effects of the bacteria *Staphylococcus aureus*, to conclude the antibiotic was ineffective against the bacteria. (‘Antibiotic resistance—the problem intensifies’, 2005)(Howden *et al.*, 2010)

Gram-negative bacterial species have a cell wall which consists of lipopolysaccharides. They are a large unique class of macromolecules found in the outer layer of the cell wall and are involved in the interaction of the cell with the external environment. Due to the permeability barrier exhibited by its external layer, gram-negative species such as *P. aeruginosa* and *E. coli* are resistant to many drugs. (Zgurskaya, López and Gnanakaran, 2015)

These Gram-negative species have been a threat to humans for a long time and have been the lead reason for admissions into the intensive care units leading to a high risk of morbidity and mortality. Organisms have developed a wide range of mechanisms to prevent and resist the action of many antimicrobials and antibacterial drugs used in clinical medicine. Some of the mechanisms of resistance include efflux pumps, alteration of the drug binding site and membrane permeability, degradation enzymes, and the conformational change of the drug culminating in its inactivation. (Oliveira and Reygaert, 2019) Herbal medicine and Ayurveda have been attracting great attention in recent years and are increasingly used as an alternative to the conventional chemical drugs available in the market. There are several publications and pieces of evidence that support the fact that medicinal plants provide a positive impact in the prevention and cure of a wide range of diseases at the same time with the benefit of providing minimum to no side effects.

*Nigella sativa* is a medical plant used for its antibacterial/antimicrobial property. One of the main components of this plant is Thymoquinone (TQ), which is the reason for its antimicrobial activity against gram-positive and gram-negative bacteria. (Ahmad *et al.*, 2013)(Ali and Blunden, 2003)

Nanomedicine is the application of nanotechnology to the field of medicine. The average size of iron oxide nanoparticles found by dynamic light scattering (DLS) particle size analyser, ranges approximately between 10 nm to 120 nm with a mean particle size of 66 nm.
Nanoparticles exert a few important attributes like nanoscale size, high surface-to-mass ratio, and high reactivity. These properties can be used to overcome most of the limitations found in common therapeutic and diagnostic agents. High amounts of bacterial toxicity mechanisms have been observed in metal-based nanoparticles which collectively makes it difficult for the development of resistance and results in broadening the array of antibacterial activity. (Dinali et al., 2017) (Tran et al., 2010)

The development of nanoparticles has been at the forefront in the last decade using a variety of processes like co-precipitation, thermal decomposition, hydrothermal synthesis, microemulsion, sonochemical synthesis, and sonochemical synthetic routes. Iron oxide nanoparticles show enhanced antibacterial activity against gram-negative bacteria, in our project we mainly focus on this property of nanoparticles. (Ali et al., 2016)

**Nigella sativa**

Herbal medicine or phytotherapy is a domain of medical science where there is the use of herbal remedies to treat the sick. It covers areas from medicinal plants with powerful actions, such as *Digitalis* and *Belladonna*, to those with very gentle actions, such as *chamomile, mint* and many others.

Medicinal plants find their applications in the manufacture of herbal medicines since it is proven to be a safer alternative to commercially available medicines. Research and analysis on a group of plants have been done for their medicinal properties, out of which *Nigella sativa* has emerged to be a herb with rich historical and traditional backgrounds. (Ali and Blunden, 2003)

*Nigella sativa* (*N. sativa*) which is cultivated in different parts of the world has been used as a remedy to treat several diseases. The seeds of this plant consist of proteins, carbohydrates, crude fibres, alkaloids, saponins, ash, fixed oils and essential oils. (Shokri, 2016). The pH of *N. sativa* seeds is around 5.6 to 5.7 or sometimes slightly lower than that which indicates its slightly acidic. (Łopusiewicz et al., 2022)
Reports have been generated on several activities of this plant, which include antioxidant and anti-inflammatory agents and furthermore used as an antihypertensive and anti-microbial agent. Studies on *N. sativa* has led to the discovery of a wide array of its pharmacological activity by multiple researchers. (Ahmad *et al.*, 2013) The seeds consist of fixed and essential oils, saponins, alkaloids and proteins, the antimicrobial activity is due to the presence of a component called Thymoquinone. The oil assists in decreasing blood pressure and increasing respiration. Rats treated with the seed extract for a period of 12 weeks showed signs of reduction in triglycerides, cholesterol and glucose. Use of either the seed extract or its oil has no impact on liver or kidney functions in the human body. (Ali and Blunden, 2003)

**Classification**

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<tr>
<td>Genus- Nigella L.</td>
<td>Nigella Specie Nigella sativa L. (Black Cumin)</td>
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</table>

Morphology of the plant

*N. sativa* is an annual flowering plant which grows up to a height of 20-90 cm with grow proliferation, flowering, seeding and death in one single season of spring and early summer. They have finely separated leaves, with diverse colours of flowers in white, yellow, pink, pale blue or pale purple, with 7-10 petals. The fruit is a large and inflated capsule composed of 4-8 united follicles each containing large amounts of seeds. *(Ahmad et al., 2013)*

Physical property of the seed

The seeds are small-scaled, bi-lobed, black externally and white internally, they have a slight aroma to them and a bitter taste. On a microscopic level, the sagittal plane of the seed depicts a mono-layered epidermis containing an elliptically thick-walled papillose cuticle composed of dark brown matter.

This is followed by two- four layers of parenchymatous cells and finally, a sepia-pigmented layer containing thick-walled rectangular lengthened cells. Endosperm comprises narrow rectangular cells mostly composed of oil spheres. *(Ahmad et al., 2013)* The chemical composition of the plant is illustrated in Fig 1.
Fig 1: Chemical Composition of *Nigella sativa* seed

**Thymoquinone**

Proteins and amino acids, carbohydrates, alkaloids, organic acids, saponins, crude fibres, vitamins and minerals are the varieties of chemical compounds found in *N. sativa* seeds. The Three-dimensional image for Thymoquinone (Fig 3) was obtained by using the SMILES ID from PUBMED, converting it to PDB and opened on the software Avogadro, to obtain a three-dimensional structure.
Thymoquinone with a chemical name of 2-isopropyl-5-methylbenzo-1, 4-quinone has been identified as a phytochemical complex found excessively in *N. sativa* seeds and oil. It was first identified and extracted by El-Dakhakhny by performing thin-layer chromatography on a silica-coated TLC plate. (‘Thymoquinone and its therapeutic potentials’, 2015)

Thymoquinone has great potential as an anti-oxidant, anti-bacterial, anti-fungal and anti-inflammatory compound. The active compounds found in these seeds are thymoquinone, thymohydroquinone, dithymoquinone (nigellone), thymol, carvacrol, nigellicine, nigellidine and α-hedrin. Thymoquinone exists in a tautomeric form and there is a presence of a keto group in high concentration due to which there are effects of pharmacological activity in them. It is a natural compound and has received high amounts of interest and focus due to its therapeutic properties. (‘Thymoquinone: Potential cure for inflammatory disorders and cancer’, 2012). However, thymoquinone as a separate biomolecule is unstable due to its poor water solubility, leading to low absorptivity and bioavailability. Therefore, it has to be dissolved in a suitable solvent like DMSO without altering its capability. (Tubesha, Bakar and Ismail, 2013)

The pharmacological activity of Thymoquinone has been illustrated in Fig 3.
Many papers support the fact that *N. sativa* prevents the formation of biofilms. Thymoquinone, a highly useful compound in *N. sativa* shows broad-spectrum activity against several strains of bacteria. For species like *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm inhibition has been observed at 22 micrograms per millilitre and 60 micrograms per millilitre. (Balyan, Shinde and Ali, 2021)

**Antifungal Activity**

*Aspergillus fumigatus* and *Aspergillus flavus* fungal growth inhibition has been analysed and proven when there is a presence of plant extract. *Trichophyton mentagrophytes*, *Microsporum gypseum*, and *Microsporum canis* are pathogenic dermatophyte strains that *N. sativa* extract has shown antifungal action against. In an in-vivo investigation, mice were infected with *Candida albicans* a pathogenic yeast present in the human gut microflora which was then treated with an aqueous extract of black seeds. The extract showed inhibiting activity towards fungal growth activity in them (Forouzanfar, Bazzaz and Hosseinzadeh, 2014) On performing a different treatment experiment for *Candida*-related infection with *Nigella sativa* extracts, the antifungal effect was assessed by measuring the zone of inhibition. These results indicated that the methanolic extracts of
Nigella sativa had a strong antifungal effect followed by the chloroform extract and the aqueous extract showed no zone of inhibition. (Bita et al., 2012)

**Antioxidant Activity**

Use of natural antioxidant compounds has gained popularity for their ability to quench their free radical to protect the human body against oxidative stress. Thymoquinone has shown promising activity against reactive oxygen species like superoxide anions and hydroxyl radicals and thus reducing their effects on the human body. The presence of quinone structure is the main reason for the anti-oxidative property shown by this compound. Thymoquinone also showed protection against several organs from oxidative damage by inducing several free radical-generating agents. (‘Thymoquinone and its therapeutic potentials’, 2015)

**Iron oxide nanoparticles**

**Size range of nanoparticles**

On average iron oxide nanoparticles have a size range of approximately 10 nm to 120 nm with a mean particle size of 66 nm (View of Synthesis and Characterization of Magnetic Iron Oxide Nanoparticles by Co-Precipitation Method at Different Conditions, no date a)

**Application of Nanoparticles**

Nanomedicine refers to the use of nanotechnology in the field of medical science. Some of the special characteristics of nanomaterials include their nanoscale size, high surface-to-mass ratio, and high reactivity. Most of the drawbacks present in frequently used medicinal and diagnostic drugs can be overcome using these characteristics. In the areas of life sciences like biomedicine, agriculture, and the environment, iron oxide has immense potential. (Sun et al., 2021)(Anjum et al., 2021)

**Iron Oxide Nanoparticles**

Iron oxide is formed by chemically combining iron and oxygen. In nature, iron (III) oxide is found in the form of rust. Generally, iron oxides are prevalent, widely used as they are inexpensive, and play an imperative role in many biological and geological processes. High amounts of bacterial toxicity mechanisms have been observed in metal-based nanoparticles which collectively make it difficult for the development of resistance and result in broadening the array of antibacterial activity. Due
to their low toxicity, superparamagnetic properties, such as surface area and volume ratio, and simple separation methodology, magnetic iron oxide (Fe₃O₄ and Fe₂O₃) NPs have attracted much attention and found their use in biomedical applications (Ali et al., 2016) The Pharmacological activity of iron oxide nanoparticles have been illustrated in Fig 4.

**Fig 4: Pharmacological properties of Iron Oxide nanoparticles**

**Anti-cancer property**

Nanoparticles' size, shape, and surface characteristics are crucial for achieving targeted anticancer activity with minimum complexity. Due to a retention effect and increased vascular permeability, nanoparticles can penetrate tumour cells with ease. Nanoparticles are frequently spherical in shape because of how easily they can be manufactured. By emitting harmless wavelength radiation that is easily absorbed by dangerous stimuli that lead to the creation of reactive oxygen species, iron oxide nanoparticles both directly and indirectly exhibit anticancer activity (Sun et al., 2021)(Anjum et al., 2021)

**Antibacterial Property**

Mutation, morphological and environmental change are the reason for the rise of multi-drug resistant bacteria. Nanoparticles with antibacterial activity have the potential to decrease the evolution of these bacterial species by targeting numerous biomolecules and preventing the formation of resistant strains. Metal-based nanoparticles have antibacterial action mechanisms which include reactive oxygen species production, cation
release and membrane interaction. Due to the lack of a sturdy layer of peptidoglycan found in the cell wall, damage caused by physical interactions between itself and the NPs is more severe in Gram-negative bacteria. Another reason for Gram-negative bacteria's vulnerability to NPs is because of the presence of a negative charge which has a greater affinity towards positive ions released by NPs which contributes to the absorption of ions which results in inner cell damage. (Humann and Lenz, 2009) Iron oxide nanoparticles show enhanced antibacterial activity against gram-negative bacteria. The main mechanism by which these particles showed antibacterial activity is because of oxidative stress generated by ROS. Damage to proteins and DNA in bacteria can be induced by ROS which includes superoxide radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen. (Wang, Hu and Shao, 2017)

**Stability of Iron Oxide Nanoparticles**

There are several issues with plane IONPs, which include limited stability, particle aggregation, fast degradation, and magnetic property changes when they are exposed directly to systems-related biological properties. The zeta potential is the electrostatic potential at the boundary between the nanoparticle's compact and diffuse layers, according to (View of Synthesis and Characterization of Magnetic Iron Oxide Nanoparticles by Co-Precipitation Method at Different Conditions, no date b; Ostolska and Wiśniewska, 2014) It is used to assess the stability of NP suspensions. If all the particles in suspension have large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to come together. Values in the -5 mV to +5 mV range imply quick aggregation, according to (Ostolska and Wiśniewska, 2014).

**Methodology**

**Preparation of Nigella sativa seed extract**

Preparation of a seed extraction by pre-treatment is an essential step in the extraction of thymoquinone by preparatory HPLC. A total of 3 different methods were used for the preparation of *N. sativa* seed extracts with various solvents. It was understood that using methanol, Hexane and aqueous extract as solvents for the process of seed extract had the highest yield of thymoquinone respectively, compared to other solvents like ethanol and benzene. (Iqbal, Ahmad and Pandey, 2018)
Pre-treatment of *N. sativa* using methanol as a solvent

Methanol is a polar solvent which is used extensively in the process of pre-treatment of *N. sativa* seeds. The seeds were dried and ground using an electric grinder by adding 10mL of water to obtain a powder. 10g of it was kept for soaking in 50 ml of methanol. The mixture was placed for stirring on a magnetic stirrer for 120mins. Filtration was performed using Whatman filter paper -1 with a pore size of 110nm. Thymoquinone is a light-sensitive compound, on filtration store the yellow filtrate in a sealed and wrapped container at 4°Celsius.

Pre-treatment of *N. sativa* using hexane as a solvent

A similar approach was used for the pre-treatment of the seeds using hexane as a solvent. The methanolic solvent was replaced with hexane and the resultant filtrate was stored in a sealed wrapped container at 4°Celsius.

Preparation of aqueous extract from *N. sativa* seeds

Aqueous extraction refers to the soaking of seeds in distilled water and using this water for further analysis. 100g of *N. sativa* seeds were dried and stored in a 500 mL glass beaker. 150mL of distilled water was added to this and soaking was done for 24hrs. The solution was then filtered using a Whatman filter paper -1 with a pore size of 110nm. On filtration, the filtrate was sealed and wrapped in a container and store at 4°Celsius.

Preparatory High-pressure liquid chromatography for extraction of Thymoquinone.

Reversed-phase preparative HPLC was carried out using Shimadzu SIL-10 AP and an LC- 20AP pump system with a UV detector at “The Bangalore Bio Innovation Centre”. The separation was performed on a Sunfire C18 column. UV detection was carried out at 254nm with an injection volume of 58.07 ml. The mobile phase was consisting of methanol as solvent A and water containing 0.1% TFA as solvent B. The gradient program set in the software is shown in Table 2.
Table: 2 Shows the concentration gradient and the time period for each.

<table>
<thead>
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<th>Time Period</th>
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<tr>
<td>50%</td>
<td>After 10 mins</td>
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<td>50%</td>
<td>Retained for 10 mins</td>
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<td>Retained for 5 mins</td>
</tr>
<tr>
<td>5%</td>
<td>5 mins</td>
</tr>
</tbody>
</table>

(Ghanavi et al., 2020)

**Synthesis of Iron oxide nanoparticles**

5.6g of Ferric chloride hexahydrate and 6.7g of ferrous sulphate were dissolved in 50ml of 0.5M HCl. 90 ml of 1.5M Sodium Hydroxide solution was added dropwise to the solution which was under vigorous mixing on a hot plate stirrer set at 80 degree Celsius and 100rpm. The pH of the solution was observed to be highly basic (pH 12), on obtaining a viscous and jet-black colouration, transfer the solution to 50 ml centrifuge
tubes. Approximately 83mL of Sodium Hydroxide was needed to obtain the viscous jet-black coloured precipitate. Centrifuge the solution at 2000 rpm for 2 minutes. Followed by the washing step where we discard the supernatant and add 10 ml of distilled water and dislodge the pellet. Perform the washing step 5-6 times until the pH of the solution is neutral (pH 6.5). Finally, discard the supernatant and transfer the pellet to a Petri plate and place it in a hot air oven set to 60 degrees Celsius for strictly 8 hours to avoid crystallization. Scrape out the powdered particles using a sterilized spatula and store them in 1.5ml tubes away from sunlight.

**Synthesis of Iron Oxide Nanoparticles using *Nigella sativa* (Methanolic extract)**

5.6g of Ferric chloride hexahydrate and 6.7g of ferrous sulphate were dissolved in 50ml of 0.5M HCl. 10mL of the prepared methanolic extract was added. 90 ml of 1.5M Sodium Hydroxide was added dropwise to the solution which is under vigorous mixing on a hot plate stirrer set at 80 degree Celsius and 100rpm. The pH of the solution was observed to be highly basic (pH 12), on obtaining a viscous and jet-black colouration, transfer the solution to 50 ml centrifuge tubes. Around 83mL of Sodium Hydroxide was needed to obtain the viscous jet-black coloured precipitate. Centrifuge the solution at 2000 rpm for 2 minutes. Followed by the washing step where we discard the supernatant and add 10 ml of distilled water. Perform the washing step 5-6 times until the pH of the solution is neutral (pH 6.5). Make sure the sides of the centrifuge tubes are sealed with Aluminium foil to prevent light exposure as Thymoquinone is light-sensitive. Finally, discard the supernatant and transfer the pellet to a Petri plate and place it in a hot air oven set at 60 degrees Celsius for strictly 8 hours to avoid crystallization. Scrape out the particles using a sterilized spatula and store it in a 1.5ml sealed tube away from sunlight.

**Synthesis of Iron Oxide Nanoparticles using *Nigella sativa* (Hexane extract)**

5.6g of Ferric chloride hexahydrate and 6.7g of ferrous sulphate were dissolved in 50ml of 0.5M HCl. 10mL of the prepared Hexane extract was added. 90 ml of 1.5M Sodium Hydroxide was added dropwise to the solution which is under vigorous mixing on a hot plate stirrer set at 80-degree Celsius. The pH of the solution was observed to be highly basic (pH 12), on obtaining a viscous and jet-black colouration, transfer the solution to 50 ml centrifuge tubes. Around 83mL of Sodium Hydroxide was needed to obtain the viscous jet-black coloured precipitate. Centrifuge the solution at 2000 rpm for 2 minutes. Followed by the washing step
where we discard the supernatant and add 10 ml of distilled water. Perform the washing step 5-6 times until the pH of the solution is neutral (pH 6.5). Make sure the sides of the centrifuge tubes are sealed with Aluminium foil to prevent light absorption as Thymoquinone is light sensitive. Finally, discard the supernatant and transfer the pellet to a Petri plate and place it in a hot air oven set at 60 degrees Celsius for strictly 8 hours to avoid crystallization. Scrape out the particles using a sterilized spatula and store it in a 1.5ml sealed tube away from sunlight. A strong odour of Nigella sativa seeds was felt during scraping.

**Synthesis of Iron Oxide Nanoparticles using *Nigella sativa* seeds (Aqueous extract)**

5.6g of Ferric chloride hexahydrate and 6.7g of ferrous sulphate were dissolved in 50ml of 0.5M HCl. 10mL of the Aqueous was added. 90 ml of 1.5M Sodium Hydroxide was added dropwise to the solution which is under vigorous mixing on a hot plate stirrer set at 80 degrees Celsius. The pH of the solution was observed to be highly basic (pH 12), on obtaining a viscous and jet-black colouration, transfer the solution to 50 ml centrifuge tubes. Around 83mL of Sodium Hydroxide was needed to obtain the viscous jet-black coloured precipitate. Centrifuge the solution at 2000 rpm for 2 minutes. Followed by the washing step where we discard the supernatant and add 10 ml of distilled water. Perform the washing step 5-6 times until the pH of the solution is neutral (pH 6.5). Make sure the sides of the centrifuge tubes are sealed with Aluminium foil to prevent light absorption as Thymoquinone is light-sensitive. Finally, discard the supernatant and transfer the pellet to a Petri plate and place it in a hot air oven set at 60 degrees Celsius for strictly 8 hours to avoid crystallization. Scrape out the particles using a sterilized spatula and store them in a 1.5ml sealed tube away from sunlight.

**Synthesis of Iron Oxide Nanoparticles using pure Thymoquinone and DMSO dissolved in Thymoquinone**

5.6g of Ferric chloride hexahydrate and 6.7g of ferrous sulphate were dissolved in 50ml of 0.5M HCl. 90 ml of 1.5M Sodium Hydroxide was added dropwise to the solution which is under vigorous mixing on a hot plate stirrer set at 80 degree Celsius at 100rpm. the pH of the solution was observed to be highly basic (pH 12), on obtaining a viscous and jet-black colouration, transfer the solution to 50 ml centrifuge tubes. Around 83mL
Author

of Sodium Hydroxide was needed to obtain the viscous jet-black coloured precipitate.

Centrifuge the solution at 2000 rpm for 2 minutes. Followed by the washing step where we discard the supernatant and add 10 ml of distilled water. Perform the washing step 5-6 times until the pH of the solution is neutral (pH 6.5). Wash it again and transfer the solution to a 150mL beaker and add 5mL of the prepared Thymoquinone or dissolve the thymoquinone in DMSO (1:1 ratio) and add 5mL of it to the iron oxide solution. Place a magnetic stirrer at 120rpm for uniform mixing (Note: No heat must be provided). Transfer the precipitate to a Petri plate and place it in a hot air oven at 60 degrees Celsius for strictly 8 hours to avoid crystallization. Scrape out the particles using a sterilized spatula and store them in a 1.5ml sealed tube away from sunlight.

Characterization Study

Characterization was performed on the size of nanoparticles obtained, zeta potential and FTIR analysis on the synthesized nanoparticles with the different extracts and the pure thymoquinone obtained by preparatory HPLC.

Size analysis by Dynamic light scattering (DLS)

The prepared particles were analysed at “Nargund College of Pharmacy, Bangalore” using the **Horiba SZ-100 nanoparticle size analyser**. The literature review suggested that the iron oxide nanoparticles are to be in the size range below 100 nm. The SZ-100 works on the principle of dynamic light scattering (DLS). DLS is the measurement of fluctuations in scattering light intensity with respect to time. These fluctuations arise due to the presence of Brownian movement of the nanoparticles in the suspension. The statistical behaviour of these fluctuations in scattering intensity can be related to the diffusion of particles. Larger particles diffuse slower than smaller ones and thus we can relate them to the particle size.
**Title**

**Relating particle size to particle motion**

This random motion is modelled by the stokes-Einstein equation. This equation connects the diffusion coefficient measured by dynamic light scattering to particle size.

\[ D_h = \frac{k_B T}{3\pi \eta D} \]

Where \( D_h \) denotes the hydrodynamic diameter of particle size, \( D_t \) represents translational diffusion coefficient \( k_B \) is Boltzmann’s constant, \( T \) is thermodynamic temperature \( \eta \) stands for dynamic viscosity.

The translational diffusion coefficient, \( D_t \), describes the tendency of a molecule to move (translational motion) under the influence of either a concentration gradient or Brownian motion. \( D_t = \kappa T / 6\pi \eta r \), and Stokes-Einstein Debye law where \( \eta \) is viscosity, \( \kappa \) is the Boltzmann constant and \( r \) is the particle’s radius. These calculations are handled by software. Temperature is constant and viscosity is displayed based on the solution.

The measurement range of particle size is from 0.3nm to 8 micrometres. The light source used in this analyzer is a He-Ne laser at 633nm. The temperature maintained inside the analyzer is 25 degrees Celsius (room temperature). The scattering angle is 90 degrees (in all four directions). A quartz cuvette cell is used to load the sample. One of the important advantages is that sample can be recovered after measurement.

**Working procedure for the apparatus**

Dilute 0.005g of the sample in 10 ml of distilled water and load the solution into the cuvette.

Place the cuvette in the loading spot on the apparatus. Run the software to obtain a graph on the average value of the size of particles present.

**Stability analysis by Zeta potential measurement**

Zeta Potential was used to analyse the stability of the nanoparticles. The prepared particles were analysed at “The Nargund College of Pharmacy” using the Horiba SZ-100 nanoparticle size analyser. The measurement range varies from -200 to +200mV. Zeta potential cells which contain
electrodes were used. Light scattering is used to determine the particle motion caused by the applied electric field. The laser light illuminates the particles, and as a result, the particles scatter light. Due to the Doppler shift, the frequency of the scattered light depends on the particle velocity. To accurately extract the frequency shift in the scattered light, a second beam of light—referred to as the reference beam—is mixed with the dispersed beam.

A small amount of suspension is added to the measuring cell before it is inserted into the instrument to take the measurement. In order to achieve the best signal-to-noise ratio, the instrument software then automatically selects the proper electric field strength, modifies the reference beam intensity, gathers and analyses the data, and displays the findings to the user. The impact of H+ or other ions on zeta potential is frequently significant.

**Working procedure for the apparatus**

Dilute 0.005g of the sample in 10 ml of distilled water and load the solution into the cuvette.

Place the cuvette in the loading spot on the apparatus. Run the software to obtain a graph and zeta potential of particles.

**Fourier-Transform infrared spectroscopy for analysis of samples**

Fourier-transform infrared spectroscopy is an analysis method to identify the presence of certain functional groups in a given sample. Analysis for the obtained sample from preparatory HPLC was tested for its functional groups using FTIR. Shimadzu FTIR-8400s apparatus was used at “Nargund college of pharmacy, Bangalore”.

*Author*
Title

Sample preparation (Liquid)

Potassium bromide (KBr) is a lead compound used in the process of FTIR. The ratio for the sample to the KBr used is 1:100. Take two stainless steel discs from the desiccator and place a piece of cardboard between the plates followed by the addition of thymoquinone ground with the KBr. Place this sandwich into a hydraulic press with a pressure of 100psi to obtain a neat clean pellet. Transfer this pallet into the sample loading spot of the machine and run the software to obtain a spectrum.

Sample preparation (Solid)

The ratio for the sample to the KBr used is 1:100. Take two stainless steel discs from the desiccator and place a piece of cardboard between the plates followed by the addition of various prepared iron oxide nanoparticles samples ground with the KBr. Place this sandwich into a hydraulic press with a pressure of 100psi to obtain a neat clean pellet. Transfer this pallet into the sample loading spot of the machine and run the software to obtain a spectrum.

Results and Discussion

Thymoquinone in Nigella sativa seeds and Iron oxide nanoparticles both show antibacterial activity against gram-negative species of bacteria. The aim of this research was the combine these two compounds to bring about a broader array of antibacterial activity against these deadly disease-causing bacteria.

Preparatory High-pressure liquid chromatography for extraction of thymoquinone

Since literature reviews suggest methanol to be the best solvent for the process of extraction preparatory HPLC was done only for this extract. On comparing the standard thymoquinone graph obtained from the literature review, the extraction of thymoquinone is at a retention time of approximately 30mins from the start of the procedure. Thymoquinone analysis is to be done at a wavelength of 254nm. A clear peak of thymoquinone was obtained at a retention time of 29mins and the resultant extract was collected in a sealed wrapped contained and stored immediately at 4°Celsius. The resultant graph has been depicted in Fig 5.
A standard graph for thymoquinone was used to compare the obtained peaks. (Ghanavi et al., 2020) On comparing it was observed that the retention time for thymoquinone is at 29 mins, thus we can conclude the extraction of thymoquinone from HPLC, a detailed FTIR analysis has been performed to confirm the presence of thymoquinone.
FTIR Analysis

Thymoquinone

The obtained graph from FTIR analysis Fig 6 were compared to the standard peaks from (Rani et al., 2018), and a comparative tabulated result was obtained in Table 3.

Fig:6 Graph obtained for the FTIR of thymoquinone extracted by preparatory HPLC

Table:3 Comparison for the values obtained and the standard values of thymoquinone

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</thead>
<tbody>
<tr>
<td>3000 cm(^{-1}) to 2800 cm(^{-1})</td>
<td>3000 cm(^{-1}) to 2800 cm(^{-1})</td>
<td>Stretching vibrations of the isopropyl and CH(_3) groups</td>
</tr>
</tbody>
</table>
On comparing the peaks and their significant, it is observed that the obtained peaks are in very close relations with the standard peaks, thus we can confirm the extracted compound to be thymoquinone.
Title

Pure Iron oxide Nanoparticles

Fig:7 FTIR graph for pure iron oxide nanoparticles

Table:4 Comparing values from literature and obtained.

<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>602 cm(^{-1})</td>
<td>617 cm(^{-1})</td>
<td>Fe-O</td>
</tr>
<tr>
<td>3398 cm(^{-1})</td>
<td>3410 cm(^{-1})</td>
<td>O-H</td>
</tr>
<tr>
<td>2359 cm(^{-1})</td>
<td>2360 cm(^{-1})</td>
<td>C-O</td>
</tr>
<tr>
<td>1621 cm(^{-1})</td>
<td>1627.98 cm(^{-1})</td>
<td>N-H</td>
</tr>
</tbody>
</table>
Methanolic Iron oxide Nanoparticles

Table: Comparing values from literature and obtained for methanolic IONP

<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>576 cm(^{-1})</td>
<td>576.74 cm(^{-1})</td>
<td>Fe-O</td>
</tr>
<tr>
<td>1100-900 cm(^{-1})</td>
<td>1107.18 cm(^{-1})</td>
<td>CH(_3)</td>
</tr>
<tr>
<td>1459.31 cm(^{-1})</td>
<td>1460 cm(^{-1})</td>
<td>CH(_3) antisymmetric bending</td>
</tr>
</tbody>
</table>
Title

| 1711.23 cm⁻¹ | 1627.98 cm⁻¹ | C=O |
| 2926.23 cm⁻¹ | 2920.33 cm⁻¹ | C-H stretching of tertiary carbon in isopropyl group. |

Hexane Iron oxide Nanoparticles

Fig: 9 FTIR graph for Hexane Iron oxide nanoparticles

Table: 6 Comparing values from literature and obtained for Hexane IONP

<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>576 cm⁻¹</td>
<td>570.95 cm⁻¹</td>
<td>Fe-O</td>
</tr>
<tr>
<td>1459.31 cm⁻¹</td>
<td>1456.31 cm⁻¹</td>
<td>CH₃ antisymmetric bending</td>
</tr>
</tbody>
</table>
Author

<table>
<thead>
<tr>
<th>2856.13 cm(^{-1})</th>
<th>2850.89 cm(^{-1})</th>
<th>Three CH(_3) groups symmetric stretching</th>
</tr>
</thead>
<tbody>
<tr>
<td>2926.23 cm(^{-1})</td>
<td>2920.33 cm(^{-1})</td>
<td>C-H stretching of tertiary carbon in isopropyl group.</td>
</tr>
<tr>
<td>1711.23 cm(^{-1})</td>
<td>Peak not present</td>
<td>C=O</td>
</tr>
</tbody>
</table>

**Aqueous Iron oxide Nanoparticles**

![FTIR for Aqueous iron oxide Nanoparticles](image)

Table: 7 Comparing values from literature and obtained for Aqueous IONP

<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavenumber (cm(^{-1}))</td>
<td>Value (cm(^{-1}))</td>
<td>Assignment</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------</td>
<td>------------</td>
</tr>
<tr>
<td>576</td>
<td>572.88</td>
<td>Fe-O</td>
</tr>
<tr>
<td>1459.31</td>
<td>1465.95</td>
<td>CH(_3) antisymmetric bending</td>
</tr>
<tr>
<td>1711.23</td>
<td>1627.98</td>
<td>C=O</td>
</tr>
<tr>
<td>2926.23</td>
<td>2922.6</td>
<td>C-H stretching of tertiary carbon in isopropyl group</td>
</tr>
</tbody>
</table>

**Thymoquinone Iron oxide nanoparticles**

![FTIR graph](image)

Fig: 11 FTIR for Thymoquinone Iron oxide Nanoparticles

Table:8 Comparing values from literature and obtained for Thymoquinone IONP
<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>576 cm(^{-1})</td>
<td>572.88 cm(^{-1})</td>
<td>Fe-O</td>
</tr>
<tr>
<td>1100-900 cm(^{-1})</td>
<td>1116.82 cm(^{-1})</td>
<td>CH(_3)</td>
</tr>
<tr>
<td>1711.23 cm(^{-1})</td>
<td>1627.98 cm(^{-1})</td>
<td>C=O</td>
</tr>
<tr>
<td>2924.81 cm(^{-1})</td>
<td>2922.26 cm(^{-1})</td>
<td>C-H stretching of tertiary carbon in isopropyl group</td>
</tr>
</tbody>
</table>

DMSO- Thymoquinone iron Oxide Nanoparticles

Fig: 12 FTIR for DMSO-Thymoquinone Iron oxide Nanoparticles
On comparing the FTIR analysis for all the following compounds, DMSO dissolved in Thymoquinone IONP showed promising results in terms of the purity and the quality of Thymoquinone present followed by the methanolic extract and hexane extract, aqueous extract IONP. Thymoquinone IONP showed the least number of functional groups which implies that the yield of thymoquinone was not efficient from these solvents.

<table>
<thead>
<tr>
<th>FT-IR (Literature)</th>
<th>FT-IR Obtained</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>576 cm(^{-1})</td>
<td>572.88 cm(^{-1})</td>
<td>Fe-O</td>
</tr>
<tr>
<td>1100-900 cm(^{-1})</td>
<td>1111.04 cm(^{-1})</td>
<td>CH(_3)</td>
</tr>
<tr>
<td>1459.31 cm(^{-1})</td>
<td>1458.24 cm(^{-1})</td>
<td>CH(_3) antisymmetric bending</td>
</tr>
<tr>
<td>1711.23 cm(^{-1})</td>
<td>1637.62 cm(^{-1})</td>
<td>C=O</td>
</tr>
<tr>
<td>2924.81 cm(^{-1})</td>
<td>2922.26 cm(^{-1})</td>
<td>C-H stretching of tertiary carbon in isopropyl group</td>
</tr>
<tr>
<td>3009 cm(^{-1})</td>
<td>3529.85 cm(^{-1})</td>
<td>CH stretching of the vinyl group</td>
</tr>
</tbody>
</table>
Nanoparticle Size Analysis

Six Samples were considered for Analysis: Iron Oxide Nanoparticles (IONP), Methanolic Extract Iron Oxide Nanoparticles (MIONP), Hexane Extract Iron Oxide Nanoparticles (HIONP), Aqueous Extract Iron Oxide Nanoparticles (AIONP), Thymoquinone Iron Oxide Nanoparticles (TIONP). The obtained results are tabulated in Table 10.

Table:10 Size analysis

<table>
<thead>
<tr>
<th>Samples</th>
<th>Size Obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>IONP</td>
<td>65.3nm</td>
</tr>
<tr>
<td>MIONP</td>
<td>63.6nm</td>
</tr>
<tr>
<td>HIONP</td>
<td>63.1nm</td>
</tr>
<tr>
<td>AIONP</td>
<td>66.8nm</td>
</tr>
<tr>
<td>TIONP</td>
<td>63.6nm</td>
</tr>
<tr>
<td>DMSO-TIONP</td>
<td>71.8nm</td>
</tr>
</tbody>
</table>
Six Samples were considered for Analysis: Iron Oxide Nanoparticles (IONP), Methanolic Extract Iron Oxide Nanoparticles (MIONP), Hexane Extract Iron Oxide Nanoparticles (HIONP), Aqueous Extract Iron Oxide Nanoparticles (AIONP), Thymoquinone Iron Oxide Nanoparticles (TIONP). The obtained results are tabulated in Table 11.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>IONP</td>
<td>12.5mV</td>
</tr>
<tr>
<td>MIONP</td>
<td>5.5mV</td>
</tr>
<tr>
<td>HIONP</td>
<td>-11.8mV</td>
</tr>
<tr>
<td>AIONP</td>
<td>-8.0mV</td>
</tr>
<tr>
<td>TIONP</td>
<td>4.9mV</td>
</tr>
<tr>
<td>DMSO-TIONP</td>
<td>15.1mV</td>
</tr>
</tbody>
</table>
Conclusion

_Nigella Sativa_ as a plant which has gained immense attention in the field of traditional medicine and has been used in India as a part of Ayurveda to treat the sick. Thymoquinone is one of the main compounds present in the seeds of this plant which is responsible for bringing about phytochemical activity in humans. Iron Oxide nanoparticles on the other hand have been used extensively in the field of medical science for their benefits of being anticancer, antibacterial, antifungal and anti-inflammatory.

Synthesizing these two natural compounds by novel methods and comparing the yield, purity and size of the compounds obtained has been the primary aim of this research. Gram-negative bacteria have been the lead microorganisms which cause diseases in humans, resistance against these microorganisms has been a prime aim of the commercially available antibiotics. They have been successful but come at a cost of several side effects.

Extraction of Thymoquinone was done using 3 different solvents namely Methanol, Hexane, and an aqueous extract to compare the yield from these 3 compounds. It was understood that methanol gave the highest yield of thymoquinone due to which HPLC was performed only for this sample.

Preparatory HPLC was carried out successfully and Thymoquinone was obtained at a retention time of 30 mins. This was further confirmed by performing FTIR for the extract and obtaining promising results for the different functional groups when compared to the available literature.

On combining all these samples with iron oxide nanoparticles FTIR was performed which confirmed the fact that Thymoquinone dissolved in DMSO IONP gave the best result followed by methanol and hexane and the least efficient extraction was by the aqueous extract. Size analysis was performed and all the samples were within the 60 to 70nm range.

Zeta Potential analysis was done to check for the stability of the samples obtained the most stable being DMSO-Thymoquinone Iron oxide nanoparticle with 15.1mV. As mentioned earlier than pH affects zeta potential values.
Since IONP is neutral the zeta potential value is higher 12.5mV which indicates stability. In MIONP, Nigella sativa seed extract is acidic (negatively charged) and the pH of methanol depends on the concentration of methanol (pH increases as the concentration increases) and the drying of the nanoparticles in a hot air oven could have decreased pH (increase in temperature reduction in pH), would have led to reduction its zeta potential to 5.5mV. (*pH in Methanol*, no date)

In HIONP Hexane is a non-polar compound and it has a neutral pH so the sample is completely acidic due to the negatively charged Nigella sativa and also the drying could have also impacted the pH, therefore, the value is - 11.8mV. Thymoquinone though neutral but because of its instability and light sensitivity and traces of impurities during HPLC extraction could have caused fluctuations in pH and reduced the zeta potential drastically leading to instability TQ IONP. The reason for adding DMSO is that it dissolves thymoquinone and it is highly alkaline which could counteract the negatively charged Nigella sativa and increase stability. The aim of the current research was to prepare stable aggregates of Thymoquinone iron oxide nanoparticles, this research can be further extended to test for their antibacterial activity on various gram-negative species of bacteria.


Anjum, S. et al. (2021) ‘Recent Advances in Zinc Oxide Nanoparticles (ZnO NPs) for Cancer Diagnosis, Target Drug Delivery, and Treatment’, Cancers, 13(18). Available at: https://doi.org/10.3390/cancers13184570.


Howden, B.P. et al. (2010) ‘Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and


