IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

BIOGENIC SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING NATURAL RESOURCE AND OINTMENT PREPARSTION.

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Abstract

Nanotechnology is a promising technology in science which have the application of synthesising a wide range of noval materials and that seems to have a huge potential in biomedical field in life science. The most exciting methods for the synthesis of nanoparticle seem to be the one which employ microorganisms and also using plant extracts. Due to the physio-chemical properties of biological based Nanoparticles, the synthesis offer an added advantage of increased life span of Nanoparticles that overcome the limitations of conventional chemical and physical methods of nanoparticle synthesis. Also the biological method will be more convenient and also cheap. The Zinc oxide (ZnO) is an inorganic compound, and one of the hardest nanoparticle which generally found in crystalline form. The ZnO is a nanoparticle which also have found a good antimicrobial activity against several pathogens (pathogenic microorganisms). In this paper, we are synthesising the ZnO nanoparticles from both Actinomycetes (from rice field) and *Cassia fistula* leaf extract by biological methods. Ana also checking its antimicrobial activities against some microorganisms and from that trying to develop an ointment using natural ingredients which promote activity against wound infecting pathogens.

Key Words : Zinc Oxide nanoparticles, Actinomycetes, *Cassia fistula*, Biological synthesis, Antimicrobial activity, Ointment.

1.0. INTRODUCTION

Nanotechnology provides promising technological tools through which a broad range of novel materials can be synthesized and that seem to have a huge potential in the field of biomedical and life sciences. Nanoparticles are considered as the starting points based on which nano structures of novel materials. One of the most exciting method for the synthesis of nanoparticle seem to be the one which employ microorganisms. Microbial process mediated nanoparticle synthesis provide materials of unusual physical, chemical, optical, and electronic properties. Biological synthesis of nanoparticles with the help of microorganisms like *Actinomycetes* has been well reported for many nanomaterials.

Actinomycetes which are commonly found in soil are a group of gram positive filamentous bacteria (Prudence *et al.,* 2020). They are well studied organisms owing to their soil degrading properties and also as a potent source of antibiotics. The mechanism that *Actinomycetes* follow to convert metallic oxides to metal oxide nanoparticles is not clearly known. Of the available biological methods for synthesis of Zinc oxide Nanoparticles. microbial methods are advantageous compared

than cell cultures or utilizing plants sources due to their scalability, efficiency and life cycle of organisms (Mohd Yusof *et al.,* 2019). This study is aimed at microbial synthesis of Zinc oxide nanoparticles from *Actinomycetes* species.

Application of Zinc oxide has been reported in several physical and optical processes including waste water treatment, food package and antimicrobial agent (Sabir *et al.,* 2014). Zinc oxide Nanoparticles are recognized as safe or GRAS, nontoxic and bio compatible particles (Zhang *et al.,* 2013). The biological method for the synthesis of Zinc oxide Nanoparticles is advantageous as it simple method and retains intact antimicrobial activity. Depending on the nature of these particles, they have a vast potential of antimicrobial activity against pathogenic microorganisms. The biological synthesis of nanoparticle is viable and cost effective and safe synthesis route according to biomedical application which confer potential and more of functional.

With all consideration scope, the study was performed to use an efficient actinomycetes mediated synthesis route to synthesize the Zinc oxide nanoparticles. One such envisage is proposed here to use the biosynthesized Zinc oxide nanoparticles as potential agent to treat as antibacterial agent against *E. coli, S. aureus P. aeruginosa and K. pneumoniae*.

Zinc oxide is one of the hardest materials, hence it does not suffer from dislocation degradation during the course of operation. Several approaches have been engaged to synthesize Zinc oxide nanocrystals such as sol–gel solvothermal, direct precipitation and hydrothermal etc.,. Most of these approaches require tedious processes, expensive substrates, sophisticated equipment and rigorous experimental circumstances. Solution combustion synthesis is one of the best and easy methods for the synthetic approach towards the uniform mixing with combustible fuel. During solution combustion, exothermic reaction between oxidizing and reducing agent takes place.

In this paper, we are the first to report the green synthesis of Zinc oxide nanoparticles via solution combustion synthesis using *Cassia fistula* leaf extract. Plant mediated synthesis of nanoparticles (NPs) is a revolutionary technique that has wide range of applications in agriculture, food industry and medicine. NPs synthesized via conventional methods have limited uses in clinical domain due to their toxicity. Due to the physio-chemical properties of plant based NPs, this method also offer an added advantage of increased life span of NPs that overcome the limitations of conventional chemical and physical methods of NPs synthesis .

Here, we report a simple and eco-friendly method of ZnO NPs synthesis from the plant extracts of *C. fistula* as reducing agents and zinc acetate as precursor for their comparative analysis of antimicrobial potential. This research will increase the potential of usage of plant based NPs in biomedical industry.

2.0. MATERIALS AND METHODS

2.1. Phase I - For Natural source 1 - *Actinomycetes*

2.1.1 Chemicals

All the chemicals used are off 99% pure AR grade from Hi Media. This includes the starch casein nitrate medium, starch casein nitrate broth, sodium hydroxide and zinc sulphate, mueller hinton agar medium. The glass materials used are off borosil grade and sterilized.

2.1.2. Collection of sample

The rhizosphere soil sampling was done based on the soil data of Kerala for rice crop growing areas indicating the richness of Zinc in the soil. In order to exploit variable microbial diversity available at various stages of crop growth, collection of rhizosphere soil samples from the agricultural field in Eravimangalam, Malappuram, Kerala fields with the coordinates 10.9444ºN, 76.2436ºE of various rice plants.

2.1.3. Isolation of *Actinomycetes*

Serial dilution of soil sample was carried out using sterile distilled water (Agadagba *et al.,* 2014) up to 10^{-6} and was plated in sterilized solid starch casein nitrate agar plates. 0.1 mL was taken from each dilution and was spread onto plates containing starch casein nitrate agar medium. The plates were incubated at 37° C for 72hrs until the appearance of *Actinomycetes* colonies. As per the study of *(*Sharma *et al.,* 2014) colonies appearing as dry, powdery and typically pigmented as green, orange, pink, yellow and white powder nature and they were for further use for future.

2.1.4. Morphological identification

Morphological identification of soil isolates is the first step in confirming the *Actinomycetes* species. It includes both naked eye observation or macroscopic observation and microscopic observation of colonies on the agar plates. Macroscopic characterization on the colonies was performed based on the following parameters such as shape, size, colour and mycelial growth. Also, the spore production was monitored based on the previous report of Muthu *et al.* (2013). Finally, the microscopic observations of important characters were see and confirmed as *Actinomycetes* (Kumar *et al.,* 2010).

2.1.5. Microbial synthesis of Zinc oxide nanoparticles

The well cultured *Actinomycetes* culture was inoculated into starch casein nitrate broth and maintained at room temperature for 4–5 days. For synthesis of Zinc Oxide nanoparticles (ZnO NPs), the samples of zinc sulphate (0.1 M) plus sodium hydroxide solution (0.4g) were taken together in a 100 mL conical flask, and 50 mL of *Actinomycete* culture was also added into the same tube and maintained in shaker incubator at 40°C for 15 min to form the ZnO nanoparticles. The flask was then heated in a microwave oven for 1–2 min followed by letting it cool for 1 h. The nanoparticles would settle down on its own in the botton of the conical flask. The appearance of white colour deposits on the bottom of the flask would confirm the formation of ZnO nanoparticles. They were subjected to synthesis of ZnO NPs. Similarly, Rajamanickam *et al.,* (2012) has used *Actinomycetes* for the biosynthesis of zinc nanoparticle for antibacterial food packaging. After this, ZnO nanoparticles were washed with deionized water and centrifugation was carried out at 3000 rpm for 10 min. The centrifugation was repeated till a clear supernatant was obtained. The pellet was collected in a small plate and it was dried in a muffle furnace at 400°C for 8 h or till it appeared totally dry. Thus ZnO nanoparticles were produced in a white powdery form as described by Mishra *et al.,* (2013).

2.1.6. Physico-chemical analysis of Zinc Oxide Nanoparticles

2.1.6.1. UV– visible spectroscopy

Biologically synthesized ZnO nanoparticles were subjected to UV–visible spectroscopy to observe the excitation spectra. It was measured using ultraviolet–visible spectrophotometer (Shimadzu, Japan), operated at the resolution of 1 nm. An absorbance spectra scan of 300–500 nm was carried out for the re-suspended nanoparticles on deionized water on the

Hitachi double beam spectrophotometer to confirm the reduction of nanoparticles (Santhoshkumar *et al.,* 2017) (Mishra *et al.,* 2013) (Dobrucka *et al.,* 2016).

2.1.6.2. Fourier transform infrared spectroscopy (FTIR)

The FTIR (Fourier transform infrared spectroscopy) was scanned for detect the binding efficiency of ZnO NPs. By employing the FTIR spectrophotometer, structural information can be elucidated from its various vibrational modes. Dried ZnO NPs powder was directly used for FTIR analysis. Scanned FTIR result was noted based on the frequency at 400–4000 cm1 with 4 cm $^{-1}$.

2.1.6.3. Scanning electron microscopy (SEM)

The surface morphology of the nanoparticles was determined using scanning electron microscopy. Zinc oxide nanoparticle were dispersed in absolute ethanol under ultrasonic stirring followed by dropping some of the solution onto the glass slide and evaporating the solvent at room temperature. Then these specimens were coated with a thin gold layer through physical vapour deposition of about 3 mm thickness in vacuum before subjecting for SEM analysis.

2.1.7. Bacterial inactivation by Zinc oxide nanoparticles

The in vitro inhibition experiment of agar well diffusion method was performed against *E. coli, S. aureus P. aeruginosa* and *K. pneumoniae* using the effect of ZnO NPs. Prepared four plates of mueller hinton agar medium was spread with staled *E. coli, S. aureus P. aeruginosa* and *K. pneumoniae* pure culture and wells were punctured into the agar using gel borer. Different concentration (25, 50 and 75 mg/mL) of ZnO NPs was inoculated into the punctured wells, and incubated overnight with 37ºC for 24hrs. After the incubation period, the zone of inhibition present around the wells of the ZnO NPs were measured. All the study carried out for bacterial inactivation of ZnO were in triplicates.

2.2. Phase II - For Natural source 2 – *Cassia fistula* plant leaf

2.2.1. Chemicals

All the chemicals used are off 99% pure AR grade from Hi Media. This includes the Folin ciocaltue reagent, Sodium carbonate, Gallic acid, Methanol, Vanillin, Hydrochloric acid, Phloroglucinol, Zinc nitrate hexahydrate, DPPH, mueller hinton agar medium. The glass materials used are off borosil grade and sterilized.

2.2.2. Collection of sample

Cassia fistula leaves were sourced from Kodikuthimala eco park, Perinthalmanna, Malappuram, Kerala, India with the coordinates 10.9827ºN, 76.2872ºE.. The plant material was shade dried and powdered into 100 mesh size and was stored at room temperature in an airtight container.

2.2.3. Preparation of the extract

1:10 proportion of the coarsely powdered plant material to water was taken in a round bottomed flask and the extraction was carried out at 100ºC with a reflux arrangement for 5 h with constant stirring. The extract was filtered and centrifuged to remove any un-dissolved material. The extract was then concentrated, dried using roto evaporator and stored in airtight bottles at 4°C.

2.2.4. Phytochemical analysis

Phytochemical examination are carried out for all extract as per the standard *methods* (Roopashree *et al.,* 2008 and obasi *et al.,* 2010).

2.2.4.1. Detection of Alkaloids

The sample (500µl) was treated with 5ml of 1% HCl solution on boiling water bath for 20 minutes. The solution was centrifuged for 10 minutes at 3000rpm and 1 ml of supernatant was treated with few drops of WAGNER'S reagent. Reddish brown colour of solution shows positive results.

2.2.4.2. Detection of Phenol

The sample (500µl) was boiled with 2ml of distilled water on water bath and filtered. 10% of ferric chloride solution was added. Blue black colour of solution positive results.

2.2.4.3. Detection of Terpenoids

The sample (2 ml) was treated with 1 ml of 2,4 di-nitrophenyl hydrazine (CNP) dissolved in 100mi of 2M HCL. Yellow orange coloration of solution shows positive result.

2.2.4.4. Detection of Tannins

The sample (500µl) was boiled with 10 ml of distilled water and filtered. In the filtrate 1M $FeCl_3$ was added. Formation of blue black precipitation shows positive result. Cooled in ice. The solution mixed with 0.5 ml of chloroform and 1ml of concentrated H_2SO_4. Formation of reddish brown ring shows a positive results.

2.2.4.5. Detection of Saponins

The sample (500µl) was boiled in 5ml of distilled water and filtered. 2.5ml of filtrate was mixed with 1.5ml of distilled water and shake vigorously. Generation of stable and persistent froth shows positive result.

2.2.4.6. Detection of Glycosides

The sample (500µl) dissolved in pyridine solution. In the solution few drops of NaOH were added. Pink to red colour shows positive results.

2.2.4.7. Detection of steroids

The sample (500µl) 2ml of chloroform and concentrated H_2SO_4 were added. In the lower chloroform layer red colour appeared that indicated the presence of steroids .

2.2.4.8. Detection of carotenoid

0.02g of sample was mixed with chloroform, mixed well and then the mixture was filtered. To the filtrate, concentrated H_2SO_4 was added, formation of blue colour at interface indicate the presence of carotenoid.

2.2.4.9. Detection of Quinine

The sample 1ml was mixed with 5ml of concentrated HCL. The formation of yellow precipitate indicates the presence of quinine.

2.2.4.10. Detection of Flavonoids

The sample (500µl) was heated (80-90°c) with 10ml of ethyl acetate over a stream bath for 3 minutes. The mixture was filtered and 4ml of filtrated was shaken with 1 ml of dilute ammonia solution and few drops of concentrated H_2SO_4 was added. Yellow coloration of the solution shows positive result.

2.2.7. Synthesis of Zinc oxide nanoparticles

ZnO nanoparticles were prepared by eco-friendly green combustion route using C. fistula plant leaf extract as fuel (A. Khorsand Zak *et al.,* 2013) , (B. Baruwati (2019)). The zinc nitrate hexahydrate (Zn (NO3)3 - 6H2O) was procured from SigmaAldrich (AR) and used without further purification. Stoichiometric amount of $Zn(NO_3)_3$ $6H_2O$ was dissolved with 0.2g of *C. fistula* leaf extract in 10ml of distilled water. The mixture was kept in a pre-heated muffle furnace at 400°C and subjected for combustion. The reaction was completed within 5 min. A fine milky white colored material was obtained. The synthesis of nano particles was repeated with different concentrations of the plant extract such as 0.3, 0.4, 0.5 and 0.6 g. The obtained product was stored in airtight container until further use.

2.2.8. Characterization

Optical properties of ZnO nanoparticles were characterized based on UV absorption spectra. The sample was sonicated for even dispersion (30 min) and the aqueous component was subsequently analysed at room temperature for optical band gap (Eg) using the UV–visible spectrophotometer (Evolution—220, Thermo Scientific).

2.2.9. Antioxidant activity

Antioxidant activity was carried out by 1, 1-Diphenyl-2- picrylhydrazyl (DPPH) assay using a modified method of Brand-Williams. DPPH (oxidized form) is a stable free radical with purple colour. In the presence of an antioxidant which can donate an electron to DPPH radical decays, and the change in absorbance at 520 nm was followed spectro-photometrically. 39.4mg of DPPH was dissolved in 100 ml of methanol to get 0.14 mM concentration of DPPH in the assay. Methanol (50%) was prepared by diluting methanol 1:1 with de-ionized water. In brief, to a 2.5 ml of 50% methanol with various concentrations like 2, 4, 6, and 8 mg of ZnO nano-powder was added and mixed with 140 ml of 1 mM DPPH solution. The mixture was incubated at 37°C for 30 min. The absorbance was recorded at 520 nm against 50% methanol blank, a control sample was maintained without addition of the test sample. The actual absorbance was taken as the absorbance difference of the control and the test sample.

2.2.10. Antibacterial activity

Antibacterial activity was screened by a agar well diffusion method against four bacterial strains namely Gram +ve bacteria *E. coli,* Gram +ve bacteria *S. aureus,* Gram +ve bacteria *P. aeruginosa and* Gram +ve bacteria *K. pneumoniae*. mueller hinton agar medium were prepared and swabbed using a sterile L-shaped glass rod with 0.1 ml of 24 h mature broth culture of individual bacterial strains. The well made by using sterile cork borer 6 mm wells was created into the each Petri-plate. Different concentrations of nano-compound (25, 50 and 75 mg/mL) were used to assess the activity of the compounds. The material was prepared in sterile water and added into the wells by using sterile micropipettes. Then the plates were incubated at 37°C for 36 h. After the incubation period, the zone of inhibition of each well was measured and the values were noted..

Phase III

2.3. Ointment preparation using Zinc oxide nanoparticle

2.3.1. Chemicals

The all chemicals used for the preparation of ointment are natural products. This includes the Honey bee wax, Honey, Avocado oil, Essential oil and the Synthesised Zinc oxide nanoparticles from both Natural souses (*Actinomycetes* and *Cassia fistula* plant leaf).

2.3.2. Collection of raw materials

The Honey bee wax and Honey were sourced from Natural honey bee culturing unit at Perinthalmanna, Malappuram, Kerala, India with the coordinates 10.9827ºN, 76.2254ºE. The Avocado oil and essential oil collected from good brand shop at Coimbatore.

2.3.3. Preparation of ointment

The collected honey bee wax were cut in to small pieces. Grate the wax base and Melt using water bath at 100ºC. Dissolve the honey bee wax in Avocado oil. After proper dissolving Add 10% of Honey. Then add ZnO NPs and dissolve in it. Add 2-3 drop of essential oil. Homogenize by continuous stir. Following the liquefaction process, the content was removed from heat and allowed the mixture to congeal. The mixture was stirred until it began to congeal. Cool just above melting point and add to Storage box. Allow to solidify. Store at Room temperature with proper labelling.

2.3.4. Evaluation of polyherbal formulations

2.3.4.1. Physical Evaluation

The colour, general appearance, and the feel on the application of the prepared formulations were noted and the results are described.

2.3.4.2. pH

The pH of the ointment formulations were determined by applying the digital type calibrated pH meter, which was further calibrated before each usage with the aid of buffered solutions at pH-7. The pH of the formulations was measured by completely dipping the reference electrode and the glass electrode into the ointment (Godbole MD *et al.,*2017) .

2.3.4.3. Spreadability

The spreadability of the formulations was determined by a special apparatus comprising of a flat wooden block supported by a pulley at its end. Based on the principle of drag and slip characteristics, the formulations were screened by placing 2 g of the polyherbal product on the ground slide. Between the two slides of the same dimensions, the formulation was sandwiched and the system was supported by a hook. To expel the entrapped air from the formulations and also to form a uniform film between the two slides, a unit kilogram weight was applied over the slide. Excess formulation extruding outside was removed from the edges. With the help of the hook, 50 g of weight was tied to induce a pulling force and the time required to cover 7.5 cm distance by the top slide was determined (Mahajan UN *et al.,* 2017). The spreadability of the formulation was determined from the formula:

2.3.4.4. Washability

The washability of the polyherbal formulations was determined by applying the ointments over the skin and the extent of easy washing with distilled water was manually observed (Mahaparale, S *et al.,* 2016).

2.3.4.5. Skin irritancy test

Over an area of 6 cm2 on the skin, 0.5 g of the formulation was applied and afterward covered with a gauze piece, which was loosely kept in contact by a dressing (semi-occlusive) for 1 hr. The gauze was removed and after 1 hr, the residual content was removed without altering other conditions. A thorough examination was performed regarding the sensitivity characteristics and other signs of rash or reaction. The protocol was performed for 7 consecutive days and grading was done (Shivhare RS *et al.,* 2018).

2.4. Comparison test for the prepared ointment

2.4.1. Antibacterial activity

Antibacterial activity was screened by a agar well diffusion method against four bacterial strains namely Gram +ve bacteria *E. coli,* Gram +ve bacteria *S. aureus,* Gram +ve bacteria *P. aeruginosa and* Gram +ve bacteria *K. pneumoniae*. mueller hinton agar medium and swabbed using a sterile L-shaped glass rod with 0.1 ml of 24 h mature broth culture of individual bacterial strains. The well made by using sterile cork borer 6 mm wells was created into the each Petri-plate. The Different wells added with ointment with nanoparticles, Same composition of ointment without nanoparticles, and respective antibiotics for each bacterial strains were used to assess the activity. The antibiotics includes Ampicillin, Vancomycin, Penicillin and Imipenem used for the bacterial strains namely Gram +ve bacteria *E. coli,* Gram +ve bacteria *S. aureus,* Gram +ve bacteria *P. aeruginosa and* Gram +ve bacteria *K. pneumoniae* respectively. The material was prepared in sterile water and added into the wells by using sterile micropipettes. Then the plates were incubated at 37°C for 36 h. After the incubation period, the zone of inhibition of each well was measured and the values were noted. The studies were conducted in triplicates and the average values were calculated for the ultimate antibacterial activity.

3.0. RESULT AND DISCUSSION

3.1. Phase I - For Natural source 1 - *Actinomycetes*

3.1.1. *Actinomycetes* culture from soil isolation

The collected soil samples were subjected to actinomycetes culture isolation. 1g of soil sample added with 10ml of distilled water. Actinomycetes colonies were isolated by serial dilution method (till 10^{-6}) using starch casein nitrate agar medium. One isolate having white aerial mycelium was isolated and they were further used for Zinc oxide nanoparticles (ZnO NPs) synthesis. The isolated colony were isolated from the soil sample colonies, weas designated as A01. The A01 culture further sub cultured and maintained as pure culture.

3.1.2. Identification of *Actinomycetes*

Microscopic examination of Gram stained actinomycetes isolates were performed. The actinomycetes exhibited purple colour under 100x magnifications which depicts the filamentous nature of gram positive bacteria. Similar studies were done to confirm the nature of actinomycetes earlier by Sharma *et al*., (2014). Muthu *et al.,* (2013) isolated *Isoptericola variabilis* from Cauvery river soil sample and confirmed them as actinomycetes by their powdery nature and stained structures.

3.1.3. Microbial synthesis of Zinc oxide nanoparticles

The microbial synthesis of ZnO nanoparticles was performed by using culturing *Actinomycetes* on starch casein nitrate broth (Fig 3.1). Microscopic identified A01 isolate, which were then evaluated for zinc oxide nanoparticle formation. The white precipitate was formed with the A01 isolate indicating the synthesis of ZnO nanoparticles (*Streptomyces enisocaesilis*). The culture extract treated with (0.1 M) zinc sulphate at 37°C for 96hr, gave a yield of 30 mg zinc oxide nanoparticles. *Actinomycetes* mediated the synthesis of ZnO nanoparticles from $ZnSO_4$.

The possible reaction for the biological synthesis is routed well below

$ZnSO_4 + 2NaOH + \text{Culture extract } Zn(OH)_2$; (White Precipitate) $+ Na_2SO_4$

$Zn(OH)_2 + 2H_2O \; Zn(OH)_4^{2-} + 2H^+$

$Zn(OH)_4^{2-} \; ZnO + 2OH + 2H_2O$

ZnOZnO NPs

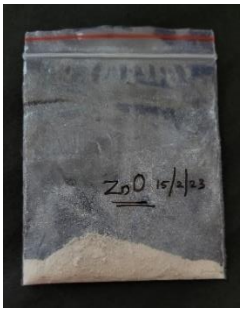

Figure 3.1 White powdery nanoparticles

3.1.4. Zinc Oxide nanoparticle characterization

3.1.4.1. UV–Visible spectroscopy analysis

The absorption spectra of Zinc oxide NPs as observed by the UV visible spectrophotometer was used as a confirmation for the presence of ZnO NPs in the colloidal solution. Based on the available colloidal nature, whether the ZnO NPs was present or not was initially confirmed by UV spectrometer and result was proved that the ZnO NPs peaks were available at 330 nm and 430 nm. The sample A01 showed peak value at 370 nm (Fig 3.2), confirmed that the synthesized product was zinc oxide nanoparticle.

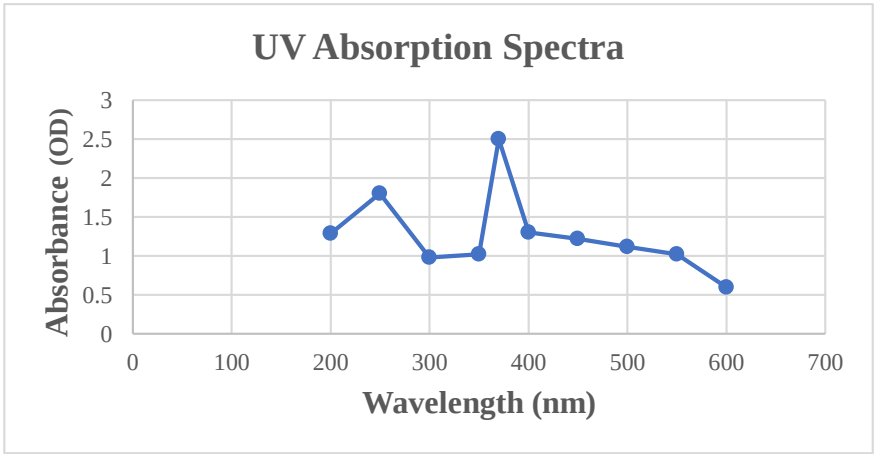

Figure 3.2 UV Absorption spectra

3.1.4.2. Fourier transform infrared spectroscopy (FTIR)

It is a well-established fact that the surface to volume ratio for the nanoparticle is higher than their bulk counterparts FTIR results would yield conclusive results on the presence or absence of various vibrational modes of synthesized ZnO NPs. The FTIR spectra of the synthesized zinc oxide nanoparticle are in the range of 4000 to 400 cm1 . The FTIR analysis was carried out for the sample A01 isolate (Fig 3.3). For sample A01 the presence of multiple peaks at 3564.77, 2993.94, 2309.34, 1757.8, 1541.81 and 1051.98, 619.038 cm1 was confirmed. The broadened appearance of intense bands with O–H bond group was confirmed at 3647 and 3564 cm1. Presence of OH peaks indicates the presence of residual moisture irrespective of heating and drying of samples. The peak bands at 1757, 1541, 1508 and 1378 cm1 represents C = O stretching of COO and CHO moiety and C-O stretching was confirmed at 1051 cm1. 619 cm1 of the bonds were confirms zinc oxide bonds. Thus the FTIR results confirm the formation of zinc oxide nanoparticle .

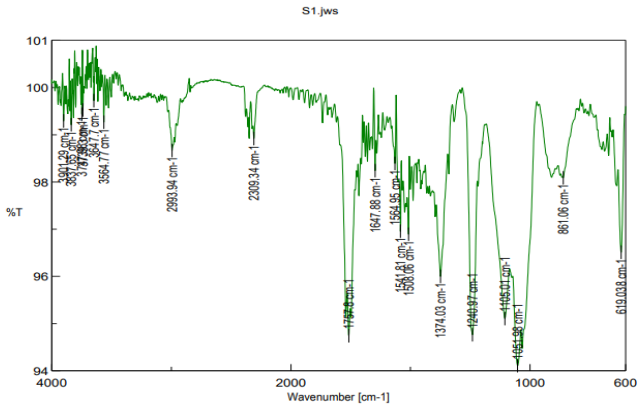

Figure 4.7 Graph of FTIR

3.1.4.3. Scanning electron microscopy (SEM)

SEM technique elucidate, A01 based ZnO NPs morphology, size and shape by SEM. The SEM image depicts that actinomycetes has enormous capacity to produce ZnO NPs that are definite with needle shape. SEM images were observed in different magnification (60,000 & 100,000). The SEM images of the synthesized ZnO NPs showed needle shaped structures of average particle size 500nm(Fig 3.4).

Figure 3.3 FTIR

Figure 4.6 Graph of UV absorption spectra

Figure 3.4 SEM

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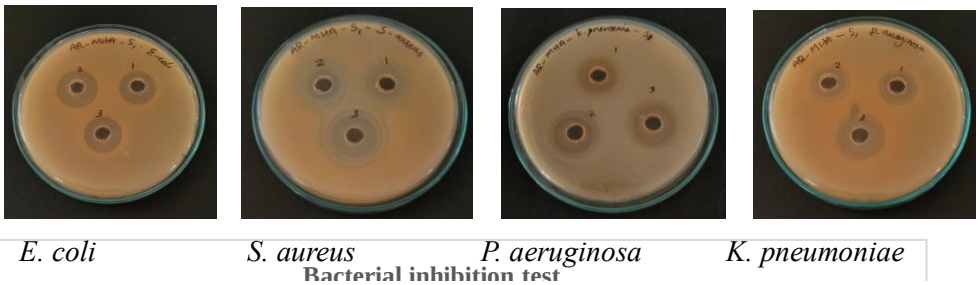
3.1.5. Biological activity analysis

3.1.5.1. Anti-bacterial activity

The Sample 1 Shows Antibacterial activity against the tested four Bacterial samples (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae)* with different range of Zone of Inhibition in Different concentration of sample (Fig 3.5 and 3.6)



E. coli *S. aureus* *P. aeruginosa* *K. pneumoniae*

Figure 3.5 Anti-bacterial activity of sample 1 ZnO nanoparticle against different micro organisms

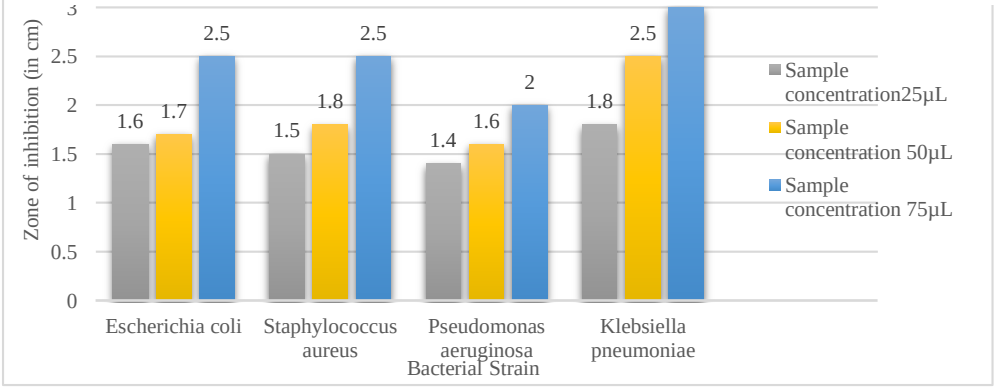


Figure 3.6 The Graphical representation of Anti-bacterial activity of sample 1

3.2. Phase II - For Natural source 2 – *Cassia fistula* plant leaf

3.2.1. Preparation of the extract

Cassia fistula plant leaf collected, cleaned and dried. The died leaf powdered. The extraction carried out using water and the extract is collected. For 200ml of water 20g of sample (leaf powder) used. The extract is dried using Rotary vacuum evaporator and hot air oven(Fig 3.7).

Figure 3.7 Extract of *Cassia fistula* leaf

3.2.2. Phytochemical analysis

The Phytochemical analysis of *C. fistula* leaf extraxt shows the presence of Tannins, Alkaloids, Flavonoids, Phenolics compounds, Saponins, Steroids, Carbohydrates, Terpenoids, Glycosides, (Table 3.1).

3.2.5. Synthesis of Zinc oxide nanoparticles

Sl. No.	Functional Groups	*Cassia fistula*
1	Tannins	Positive
2	Alkaloids	Positive
3	Flavonoids	Positive
4	Phenolics compounds	Positive
5	Quinones	Negative
6	Saponins	Positive
7	Steroids	Positive
8	Carbohydrates	Positive
9	Terpenoids	Positive
10	Glycosides	Positive

Table 3.1 The Phytochemical analysis of *Cassia fistula* leaf extract

The synthesis of Zinc oxide nanoparticles by eco-friendly green combustion route using C. fistula plant leaf extract as fuel. The zinc nitrate hexahydrate is added with cassia fistula extract and ZnO nanoparticles synthesised by Green combustion. After the combustion a fine milky white colored material was obtained.

3.2.6. UV–Visible spectroscopy analysis

The absorption spectra of Zinc oxide NPs as observed by the UV visible spectrophotometer was used as a confirmation for the presence of ZnO NPs in the colloidal solution. Based on the available colloidal nature, whether the ZnO NPs was present or not was initially confirmed by UV spectrometer and result was proved that the ZnO NPs peaks were available at 330 nm and 430 nm. The sample showed peak value at 360 nm (Fig 3.8), confirmed that the synthesized product was zinc oxide nanoparticle.

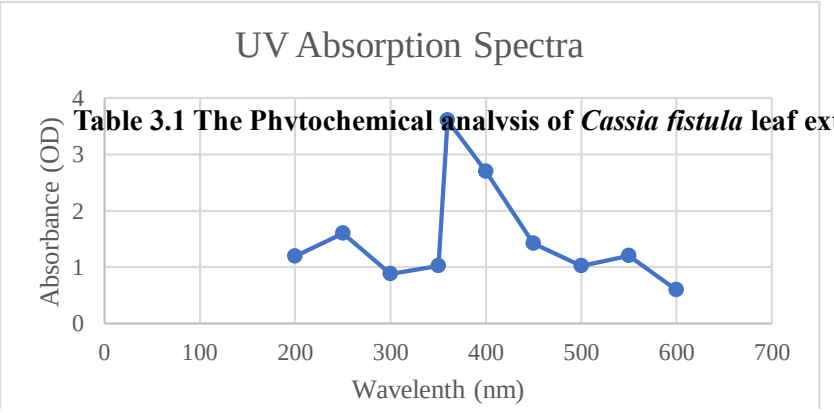


Figure 3.8 The graphical representation of UV absorption spectra

3.2.7. Anti-oxidant activity

The OD values of Sample 2 in different concentration Shows the almost same as the standard values in Antioxidant DPPH assay (Fig 3.9).

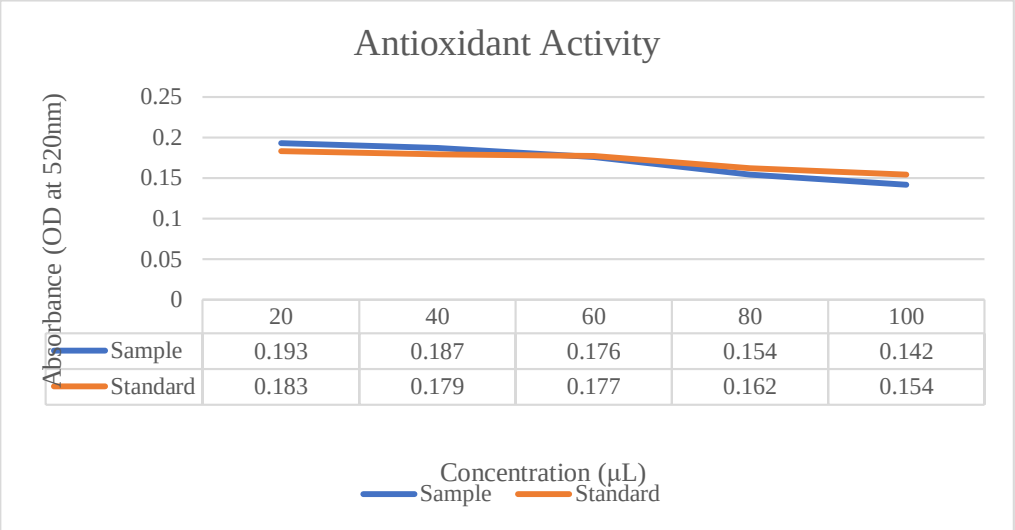


Figure 3.9 The graphical representation of DPPH assay

$$\text{Anti oxidant activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

$$= \frac{0.398 - 0.412}{0.398} \times 100$$

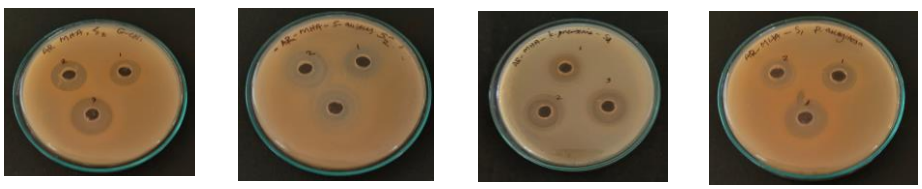
$$= 64\%$$

The sample shows 64% of antioxidant activity. Antioxidant activity was examined using a DPPH free radical assay. ZnO NPs demonstrated moderate antioxidant activity by scavenging 45.47% DPPH at 1mg/mL (P C Nagajyothi *et al.,* 2015)

3.2.8. Biological activity analysis

3.2.8.1. Antibacterial activity

The Sample 2 Shows Antibacterial activity against the tested 4 Bacterial samples with different range of Zone of Inhibition in Different concentration of sample (Fig 3.10 and 3.11)



E. coli *S. aureus* *P. aeruginosa* *K. pneumoniae*

Figure 3.10 Anti-bacterial activity of sample 2 ZnO nanoparticle against different microorganisms

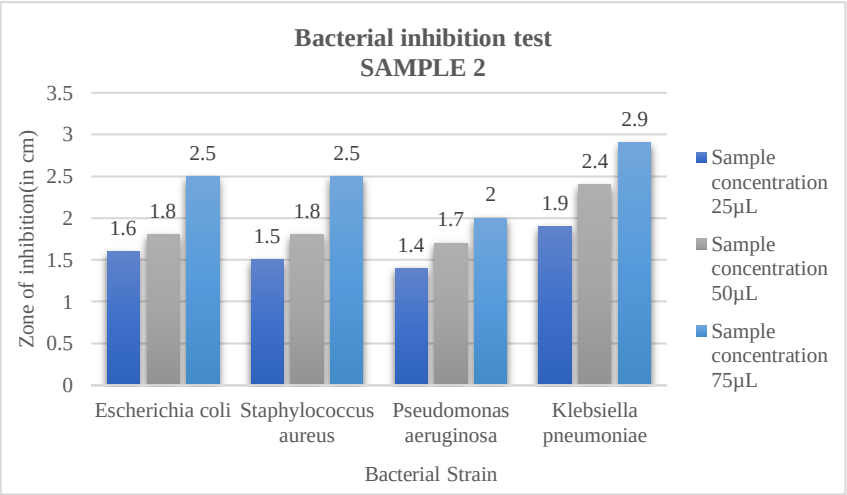


Figure 3.11 The Graphical representation of Antibacterial activity of sample 2

3.3. Ointment preparation using Zinc oxide nanoparticle

3.3.1. Evaluation of formulations

Sl. No	Ingredients	Activity of Ingredient	Amount used
1	ZnO Nanoparticle S1	Antibacterial	0.5ml
2	ZnO Nanoparticle S2	Antibacterial	0.5ml
3	Honey Bee wax	Base	14g
4	Avocado oil	Moisturizing , Vitamin E	50ml
5	Essential oil	Anti inflammatory	2-3 drops
6	Honey	Wound healing, Antiseptic	1ml

Figure 3.12 Prepared of ointment.

3.3.2. Physical Evaluation

The ointment formulations look very elegant, colored, very soft to touch, free from grittiness, non-irritant, and no such defects detected. The ointment has a golden yellow colored appearance with characteristic pleasant-type odor (Table 3.2).

3.3.3. pH

The pH of the formulations was found to be 6, which is ndicated compatibility for dermal application as the formulation pH closely lies with the pH of the skin (5.4- 6.0).

Table 3.2 The Physical Evaluation of ointment

Sl. No.	Parameters	In Ointment
1	Temperature	Room Temperature
2	pH	6
3	Appearance	Golden yellow colour
4	Consistency	Semi solid, Smooth. Soft
5	Odour	Pleasant
6	Spreadability	Easy spreadable(4.6 g.cm/sec)
7	Type of Smear	Greasy
8	Homogeneity	Good
9	Washability	Moderate
10	Irritation test	Non irritable

3.3.4. Spreadability

The spreadability of ointment formulations was found to be 4.6 g.cm/sec. As the viscosity of the developed formulation decreases, the spreadability increases concurrently. The higher activity of ointment may be due to less spreadability and more retention to the wounded area, which causes a concentrated amount of the product in the focused area.

3.3.5. Washability

The washability of the polyherbal formulations was determined by applying the ointments over the skin and the extent of easy washing with distilled water was manually observed as moderate level.

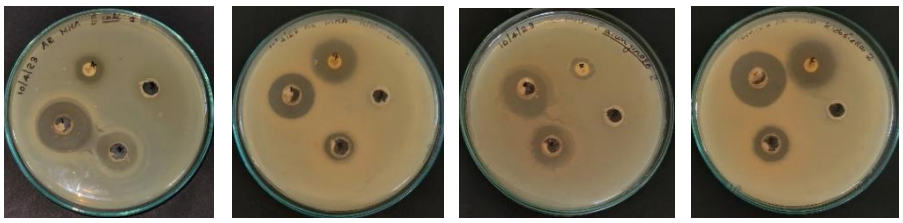
3.3.6. Skin irritancy test

From the skin irritation test study, no particular edema or erythema symptoms were seen after application for consecutive 7 days. From simple observation, it was noticed that the ointment was non-irritant. While looking at the compatibility of usage, numerous synthetic cosmetics in the market contain new synthetic excipients, which lead to skin irritation in sensitive populations, in contrast to it, polyherbal formulations demonstrated better compatibility for human use with no local irritation.

3.4. Comparison test for the prepared ointment

3.4.1. Antibacterial activity

The Antibacterial activity against the tested 4 Bacterial samples with different range of Zone of Inhibition in Different type of samples (Fig 3.13 and 3.14). It shows more antibacterial activity to the Ointment with NPs compared to the ointment without NPs.



E. coli *S. aureus* *P. aeruginosa* *K. pneumoniae*

Figure 3.13 Zone of Inhibition of comparison test

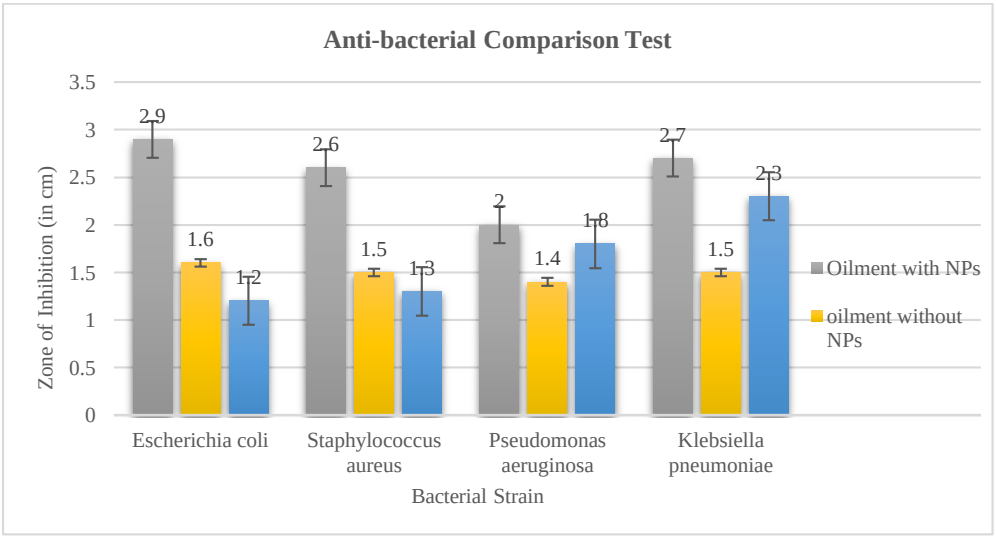


Figure 3.14 The Graphical representation of Anti-bacterial activity of different type of samples

5.0. SUMMARY AND CONCLUSION

Synthesis of Zinc Oxide Nanoparticles for biomedical application is safe using biological synthesis by using natural resouces such as *Actinomycetes* and *Cassia fistula* plant leaf . It can be used to improve the drug against the wound infecting micro organisms. The ZnO NPs being a stable structure and safe compound can be used as dental fillers with more activity of biofilm degradation that is stimulates the cavity development. Biological mediated synthesis confers safe route of synthesis for biomedical application. The present study has envisaged the activity of the biologically synthesized ZnO nanoparticle as an alternative compound against the micro organisms such as *E. coli, S. aureus P. aeruginosa and K. pneumoniae* . The study also describe the preparation of herbal ointment in corporate with Zinc oxide nanoparticle which shows the anti bacterial properties. The ointment shows more Antibacterial activity than Ointment without NPs. In future it can act as a safe biological and potential compound for safe and exact for Wound Infection.

REFERENCE

1. A. Khorsand Zak, W.H. Abd. Majid, M.R. Mahmoudian, Majid Darroudi, Ramin Yousefi, *Adv. Powder Technol*. Starch-stabilized synthesis of ZnO nano powders at low temperature and optical properties study 24 (2013) 618–624.
2. A.B.D. Nandiyanto, et al. How to Read and Interpret FTIR Spectroscope of Organic Material. *Journal of Science & Technology*, Volume 4 Issue 1, April 2019.
3. Agadagba et al. (2014) Isolation of Actinomycetes from Soil *Journal of Microbiology Research* 2014, 4(3): 136-140
4. Ahmed, S., Ahmad, M., Swami, B. L. & Ikram, S. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: A green expertise. *J. Adv. Res.* 7, 17–28 (2016).
5. Al-Dhabi, N. A. & Arasu, M. V. Environmentally friendly green approach for the production of Zinc oxide nanoparticles and their anti-fungal, ovicidal, and larvicidal properties. Nanomaterials 8, 500 (2018).
6. Arya, V. Living systems: Eco-friendly nanofactories. *Dig. J. Nanomater Bios*. 5, 9–21 (2010).
7. Avish d maru *et al.,* (2019) Formulation and evaluation of ointment containing sunflower wax. *Asian J Pharm Clin Res*, Vol 12, Issue 8, 2019, 115-120
8. Awwad, A., Albiss, B. & Ahmad, A. L. Green synthesis, characterization and optical properties of zinc oxide nanosheets using Olea europea leaf extract. *Adv. Mater. Lett*. 5, 520–524 (2014).
9. Azam, M. M., Mamoon-ur-Rasheed, A. N. M., Towfque, N. M., Sen, M. K. & Nasrin, S. Pharmacological potentials of Melia azedarach L. - *A review. Am. J. Bios*. 1, 44–49 (2013).
10. B. Baruwati, Green Chem. Glutathione promoted expeditious green synthesis of silver nanoparticles in water using microwaves 11 (2009) 926–930
11. Bahorun, T., Neergheen, V. S. & Aruoma, O. I. Phytochemical constituents of Cassia fstula. Afri. *J. Biotechnol*. 4, 1530–1540 (2005).
12. Balagurunathan, R., and Radhakrishnan. M. 2007.Actinomycetes: Diversity andtheir importance. In: Microbiology Applications and Current Trends. P.C. Trivedi (editor), *Pointer publishers*, Jaipur, India.pp. 297-329
13. Baliah, N.T., M, P., Priyatharsini, S.L., 2018. Synthesis and characterization of onion mediated silver doped zinc oxide nanoparticles. *Int. J. Sci. Res. Sci. Eng. Technol*. 4, 111–120. https://doi.org/10.32628/ijsrset11841129.
14. Balraj, B., Senthilkumar, N., Siva, C., Krithikadevi, R., Julie, A., Potheher, I.V., Arulmozhi, M., 2017. Synthesis and characterization of Zinc Oxide nanoparticles using marine streptomyces sp. with its investigations on anticancer and antibacterial activity. *Res. Chem. Intermed*. 43 (4), 2367–2376. https://doi. org/10.1007/s11164-016-2766-6.
15. Bhatte, K. D., Sawant, D. N., Pinjari, D. V., Pandit, A. B. & Bhanage, B. M. One pot green synthesis of nano sized zinc oxide by sonochemical method. *Mater. Lett*. 77, 93–95 (2012).
16. Biotechnol. 10, 1–22. https://doi.org/10.1186/s40104-019-0368-z.
17. C. Perez, M. Paul, P. Bazerque, Acta. Biol. Med. Exp. 15 (1990) 113–115.
18. Chandra Pratap Singh et al., (2016) Design and Formulation of Tridax procumbens based Polyherbal Cream for Wound Healing Potential. *Der Pharmacia Lettre.*
19. Chao Wang, Antibacterial effects of zinc oxide nanoparticles on Escherichia coli K88, *Afr. J. Biotechnol*. 11 (2012) 10248–10254, doi:10.5897/AJB11.3703.
20. D. Suresh, Udayabhanu, P.C. Nethravathi, K. Lingaraju, H. Rajanaika, S.C. Sharma, H. Nagabhushana, Spectrochim. *Acta A* 136 (2015) 1467–1474

21. Das, D., Nath, B. C., Phukon, P. & Dolui, S. K. Synthesis and evaluation of antioxidant and antibacterial behavior of CuO nanoparticles. Colloids. Surf. B Biointerfaces. 101, 430–433 (2013).
22. Datta, A., Patra, C., Bharadwaj, H., Kaur, S., Dimri, N., Khajuria, R., 2017. Green synthesis of zinc oxide nanoparticles using parthenium hysterophorus leaf extract and evaluation of their antibacterial properties. *J. Biotechnol. Biomater*. 07, 3–7. https://doi.org/10.4172/2155-952x.1000271.
23. Dobrucka, R., Długaszewska, J., 2016. Biosynthesis and antibacterial activity of ZnO nanoparticles using trifolium pratense flower extract. *Saudi J. Biol. Sci*. 23 (4), 517–523. https://doi.org/10.1016/j.sjbs.2015.05.016.
24. Fan, Z. Y. & Lu, J. G. Zinc oxide nanostructures: Synthesis and properties. *J. Nanosci. Nanotechnol*. 5, 1561–1573 (2005).
25. Godbole MD, Mahapatra DK, Khode PD. Fabrication and characterization of edible jelly formulation of stevioside: a nutraceutical or OTC aid for the diabetic patients. *Inventi Nutraceut*. 2017;2017(2):1-9.
26. Gunalan, S., Sivaraj, R. & Rajendran, V. Green synthesized ZnO nanoparticles against bacterial and fungal pathogens. *Progress in Natural Science: Materials International* 22, 693–700 (2012).
27. H. Mirzaei, M. Darroudi, Zinc oxide nanoparticles: biological synthesis and biomedical applications, *Ceram. Int*. 43 (2017) 907–914, doi:10.1016/j.ceramint. 2016.10.051.
28. H. Raja Naika, K. Lingaraju, K. Manjunath, Danith Kumar, G. Nagaraju, D. Suresh, H. Nagabhushana, *J. Taibah Univ. Sci*. 9 (2015) 7–12.
29. H.F. McMurdie, M.C. Morris, E.H. Evans, B. Paretzkin, W. Wong-Ng, L. Ettlinger, C.R. Hubbard, *Powder Diffr*. 1 (1986) 64–77.
30. Ibrahem, E.J., Thalij, K.M., Saleh, M.K., Badawy, A.S., 2017. Biosynthesis of zinc oxide nanoparticles and assay of antibacterial activity. *Am. J. Biochem. Biotechnol.* 13 (2), 63–69. https://doi.org/10.3844/ajbbsp.2017.63.69.
31. Jeffrey, L.S. (2008) Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak. *Afr. J. Biotechnol*., 7: 3697-3702.
32. Jivad N, Bahmani M, Asadi-Samani M. A review of the most important medicinal plants effective on wound healing on ethnobotany evidence of Iran. *Der Pharmacia Lettre*. 2016;8(2):353-7.
33. Kalpana, V. N. & Rajeswari, V. D. A review on green synthesis, biomedical applications, and toxicity studies of ZnO NPs. Bioinorg. *Chem. Appl*. https://doi.org/10.1155/2018/3569758(2018).
34. Kavitha, K. S. et al. Plants as green source towards synthesis of nanoparticles. *Int. Res. J. Biol. Sci*. 2, 66–76 (2013)
35. Khalil, M.I., Al-Qunaibit, M.M., Al-zahem, A.M., Labis, J.P., 2014. Synthesis and characterization of ZnO nanoparticles by thermal decomposition of a curcumin zinc complex. *Arab. J. Chem*.
36. Kumar, N., Singh, R.K. and Mishra, S.K. et al. (2010) Isolation and screening of soil actinomycetes as sources of antibiotics active against bacteria. *International Journal of Microbiology Research*, 2: 12 – 16.
37. Mahajan UN, Mahapatra DK, Mahajan NM, Kazi FS, Baghel N. Exploring the role of Mahua oil as potent emulsifier in cream formulations. *Int J Herb Med*. 2017;5(3):93-7.
38. Mahaparale, S., Gaikwad, A. Formulation and evaluation of a novel non steroidal anti inflammatory zaltoprofen gel. *World J Pharm Pharm Sci*, 2016; 5(7): 1327-1335.
39. Makarov, V. V. et al. "Green" nanotechnologies: synthesis of metal nanoparticles using plants. *Acta Naturae* 6, 35–44 (2014).
40. Malik, P., Shankar, R., Malik, V., Sharma, N. & Mukherjee, T.K. Green chemistry based benign routes for nanoparticle synthesis. *J. Nanopart*. https://doi.org/10.1155/2014/302429(2014).

41. Maruthupandy, M., Rajivgandhi, G., Muneeswaran, T., Song, J.M., Manoharan, N., 2018. Biologically synthesized zinc oxide nanoparticles as nanoantibiotics against ESBLs producing gram negative bacteria. *Microb. Pathog.* 121, 224–231. https://doi.org/10.1016/j.micpath.2018.05.041.
42. Minha Naseer *et al.,* (2020) Green route to synthesize Zinc Oxide Nanoparticles using leaf extracts of Cassia fstula and Melia azadarach and their antibacterial potential. 10:9055 | https://doi.org/10.1038/s41598-020-65949-3
43. Mishra, M., Paliwal, J.S., Singh, S.K., Selvarajan, E., Subathradevi, C., Mohanasrinivasan, V., 2013. Studies on the inhibitory activity of biologically synthesized and characterized zinc oxide nanoparticles using lactobacillus sporogens against *staphylococcus aureus*. *J. Pure Appl. Microbiol*. 7, 1263–1268.
44. Mittal, A. K., Chisti, Y. & Banerjee, U. C. Synthesis of metallic nanoparticles using plant extratcs. *Biotechnol*. Adv. http://dx.doi. org/10.1016/ (2013).
45. Mohd Yusof, H., Mohamad, R., Zaidan, U.H., Abdul Rahman, N.A., 2019. Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: A review. *J. Anim. Sci.*
46. Multimer M. Spreadability determination by an apparatus. *J Am Pharm Assoc* 1956;45:212-4.
47. Muthu, M.R., Subbaiya, R., Balasubramanian, M., Ponmurugan, P., 2013. Isolation and identification of actinomycetes isoptericola variabilis from cauvery river soil sample. *Int. J. Curr. Microbiol. App. Sci* 2, 236–245.
48. Nagori BP, Solanki R. Role of medicinal plants in wound healing. *Research Journal of Medicinal Plant*. 2011;5(4):392-405.
49. P C Nagajyothi, Sang Ju Cha, In Jun Yang, T V M Sreekanth, Kwang Joong Kim, Heung Mook Shin. Antioxidant and anti-inflammatory activities of zinc oxide nanoparticles synthesized using Polygala tenuifolia root extract. *J Photochem Photobiol B*. 2015 may;146:10-7. DOI: 10.1016/j.jphotobiol.2015.02.008
50. Pattanayak S, Nayack SS, Dinda SC, Panda D, Naval KP. Evaluation of herbal ointments formulated with methanolic extract of Cajanus scarabaeoides. *J Pharm Allied Health Sci* 2011;2:47-59.
51. Prudence, S.M.M., Addington, E., Castaño-Espriu, L., Mark, D.R., Pintor-Escobar, L., Russell, A.H., McLean, T.C., 2020. Advances in actinomycete research: An actinobase review of 2019. *Microbiol. (United Kingdom)* 166, 683–694. https://doi.org/10.1099/mic.0.000944.
52. R. Navale, D.J. Late, S.S. Shinde, JSM Nanotechnology & nanomedicine antimicrobial activity of ZnO nanoparticles against pathogenic bacteria and fungi, *JSM Nanotechnol. Nanomed*. 3 (2015).
53. Raina R, Prawez S, Verma PK, Pankaj NK. Medicinal plants and their role in wound healing. *Vet Scan*. 2008 Jan;3(1):1-7.
54. Rajamanickam et al., (2012) biosynthesis of zinc oxide nanoparticles and its characterization for antibacterial and anticancer properties. *World Journal of Pharmacy and Pharmaceutical Sciences*
55. Rajasree PH, Vishwanad V, Cherian M, Eldhose J, Singh R. Formulation and evaluation of antiseptic polyherbal ointment. *International Journal of Pharmacy & Life Sciences.* 2012 Oct 1;3(10).
56. Ruchi S. Shivhare et al., (2019). Development of Wound Healing Ointment Formulation containing Active Extracts of Tridax Procumbens, Calendula Officinalis, Murraya Koenigii, and Aloe Barbadensis. *International Journal of Pharmaceutical and Phytopharmacological Research (eIJPPR)*
57. Sabir, S., Arshad, M., Chaudhari, S.K., 2014. Zinc oxide nanoparticles for revolutionizing agriculture: Synthesis and applications. *Sci. World J.* 2014, 1– 8. https://doi.org/10.1155/2014/925494.

58. Santhoshkumar, J., Kumar, S.V., Rajeshkumar, S., 2017. Synthesis of zinc oxide nanoparticles using plant leaf extract against urinary tract infection pathogen. Resour. *Technol*. 3 (4), 459–465. https://doi.org/10.1016/j.reffit.2017.05.001.
59. Sharma, M., Dangi, P., Choudhary, M., 2014. Actinomycetes : Source. Identification, and Their Applications 3, 801–832.
60. Shivhare RS, Kamble MA, Mahapatra DK, Ingole AR, Baheti JR. Development of mosquito repellant gel formulations from various natural volatile oils: comparative study with the marketed formulation odomos®. *Journal of Drug Delivery and Therapeutics.* 2018 Nov 15;8(6):106-10.
61. Shkodenko, L., Kassirov, I., Koshel, E., 2020. Metal oxide nanoparticles against bacterial biofilms: Perspectives and limitations. Microorganisms 8, 1–21. https://doi.org/10.3390/microorganisms8101545.
62. Singleton, V.L. and Rossi, J.A. (1965) Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagent. *American Journal of Enology and Viticulture,* 16, 144-158. http://www.ajevonline.org/content/16/3/144.full.pdf+html
63. Swain, T. and Hillis, W.E. (1959) The Phenolic Constituents of Prunus domestica. I.—The Quantitative Analysis of Phenolic Constituents. *Journal of the Science of Food and Agriculture*, 10, 63-68. http://dx.doi.org/10.1002/jsfa.2740100110
64. Veerachari, U. & Bopaiah, A. K. Preliminary phyto-chemical evaluation of the leaf extract of fve cassia species. *J. Chem. Pharm*. Res. 3, 574–584 (2011).
65. W. Brand-Williams, Food Sci. *Technol*. 28 (1995) 25–30. [32]
66. Zhang, Y., Nayak, T.R., Hong, H., 2013. Biomedical applications of zinc oxide nanomaterials. *Curr Mol Med*. 13.