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GREEN SYNTHESIS AND CHARACTERIZATION OF ZNO NANOPARTICLES FROM PHYLLANTHUS NIRURI AND THEIR INCORPORATION INTO BIOFILMS FOR ACTIVE FOOD PACKAGING

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Abstract

Green nanotechnology is gaining a lot of attention as a result of the growing need for non-toxic and sustainable materials in food packaging. This work used Phyllanthus niruri leaf extract as a natural reducing and stabilizing agent to create zinc oxide nanoparticles (ZnO NPs) in an environmentally friendly manner. The formation, crystalline, functional groups, and surface shape of the bio synthesized ZnO NPs were confirmed by means of UV-Vis spectroscopy, FTIR, XRD, and SEM. The nanoparticles had favorable physicochemical characteristics that made them appropriate for use in antibacterial applications. The ZnO NPs were then added to a matrix made of bio polymers to create a biofilm that might be used in active food packaging. The mechanical characteristics, water vapor permeability, and antibacterial efficacy against food borne pathogens of the produced biofilms were assessed. The potential of ZnO NP-enriched biofilms as an efficient active packaging material was shown by the results, which showed improved barrier qualities and increased antibacterial activity. In addition to extending food safety and shelf life, this environmentally friendly strategy supports plant-based nanotechnology-based sustainable packaging solutions.

Keywords: Green nanotechnology, Phyllanthus niruri, Antimicrobial,biofilm, food packaging

Introduction

Nanotechnology is a branch of engineering and science that focuses on creating incredibly small structures and gadgets. These gadgets are used in many different fields, including electronics, medicine, energy, textiles, and packaging. Materials with overall dimensions below 100 nm, or the nano scale, are called nanoparticles. People have been quite interested in nanoparticles that come in different sizes, compositions, and shapes that stay the same. Nanotechnology and nano-science are rapidly expanding fields with a wide range of real-world uses, including antibacterial agents [1], biochemical industries, manufacturing, environmental safety, waste water treatment [2] and food additives [3]

Various techniques, such as phy~~sical, chemical,~~ sicochemical and ~~biological/~~green synthesis ~~,~~ were used to create nanomaterials~~particles~~ [4]. Most significantly

used methods are Lithography, ssonication, electrospinning etc. The ~~, and other physical synthesis techniques are examples.~~ Chemical methods like~~,~~ ~~such as~~ sol-gel, photochemical reduction, molecular condensation, and electrochemical modifications~~,~~ are frequently used to form~~eate~~ and stabilizes the metallic nanoparticles. Due to unforeseen consequences like ~~Chemical methods of synthesis have unforeseen consequences, including~~ contamination ~~of the environment, s,~~ high energy consumption, the chemical synthesis leads to ~~and~~ cause possible health issues. Hence green synthesis is the best method for reparation of nanomaterials~~Zno nanoparticles are best synthesized via green synthesis~~ [5]. ~~In order to minimize metal ions, green synthesis (Jie Hong et al., 2022) uses plant extracts rather than industrial chemical agents. It is based on microbes, plants, and biomimicry (proteins, cells, pollens, and enzymes). G~~Green synthesis is the mo~~stre~~ advantageous process than traditional chemical synthesis since it is less harmful, cost effective~~less expensive , produces less pollution,~~ and enhances the safety of the environment and human health [6]

~~The tiny erect annual herb~~ Phyllanthus niruri is a tiny herb grown in ~~indigenous~~ tropical and subtropical areas around the world, it belongs to ~~to the Amazon rainforest and other tropical regions like China, South East Asia, and Southern India. In height, it can reach 30 to 40 centimeters. It has 7–12 cm long, alternating sessile and oblong leaves. T~~the family Euphorbiaceae ~~family. includes this herb, which is widely distributed worldwide.~~ Numerous biologically active ~~physiologically active~~ substances were ~~, including astragalin, corilagin, lintetralins, phyllanthine, phyllochrysine, quercetol, rutin, triacontanal, quercetin, lignans, carboxylic acids, ellagitannins, geranin, brevifolin, alkaloids, methylsalicylate, phyltetralin, saponins, acisquercitrin, phyllanthenol, tricontanol, and repandusinic are among the biologically active substances~~ found in Phyllanthus niruri[7]. ~~The plant extract from~~ Phyllanthus niruri is used to cure a variety of ailments, including bronchitis, constipation, ~~kidney stones~~nephrolithiasis, and excess uricacidation. ~~Jaundice, skin conditions, diabetes, chest pain, and ulcers were among the many medicinal applications of bhumi amla.~~ In addition, ~~to efficiently treating hepatitis,~~ this plant extract ~~may also~~ used to treat ~~AIDS~~HIV, ringworm, hemorrhoids, ~~, infection with the HIV virus,~~ hepatitis B~~, and anti-cancer activity, wound healing, and anti-ulcer qualities~~ [8]. By considering all of these therapeutic benefits, Phyllanthus niruri was used to create ZnO nanoparticles

The increasing environmental concerns associated with conventional plastic packaging have spurred the development of biodegradable polymer-based materials for sustainable food packaging solutions. Biodegradable polymers such as chitosan, starch, gelatin, cellulose, **poly lactic** acid (PLA), and alginate have gained attention due to their eco-friendly nature, biocompatibility, and ability to degrade under natural conditions. However, these materials often exhibit limitations such as poor mechanical strength, high water permeability, and limited antimicrobial properties, which restrict their broader application in food packaging.

In this current study we used aqueous extract of Phyllanthus niruri for green synthesis of ~~e~~ Zinc oxide nanoparticles (ZnO-NPs) for active food packaging. Food packaging is commonly employed to protect food from physical and chemical damages, extending shelf life by avoiding microbial growth and oxidative spoilage's. ZnONPs are fast growing emerging services in active food packaging. Because of their nano size, broad spectrum antibacterial activity against food born pathogens and UV-blocking properties ~~are tiny particles with powerful **antimicrobial** (Sirelkhatim, A, 2015) and **UV-blocking** properties, making them~~

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ideal for **food packaging**. They help extend shelf life by preventing microbial growth and protecting against moisture, oxygen, and lightthe . ZnO-NPs are often blended into biodegradable polymers to create active food packaging. films. ZnO nanoparticles have demonstrated antibacterial qualities and possible uses in food preservationTo give the packaging material antibacterial activity and enhance the certain packaging qualities, the synthesized ZnO nanoparticles washave been added to starch polymeric matrix [9].). The nanobased starch polymers are biodegradable active packaging criteria than Pepetroleum based polymers which are the primary materials used in the packaging industry. Hence we planned to designed a biodegradable nano based food packing film with more shelf life and less toxicity in Vero cell lineThe environmental, ecological and safety concerns over petroleum-based **polymers have raised a global trend in the use of biodegradable polymers.**

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Methadology

Collection and Preparation of Aqueous extractprocessing of plant sample:

The aqueous extract was prepared by mixing the 10gms of Phyllanthus niruri leaves powder in 100 ml of distilled water for 30 minutes at 70ºC. After incubation, the filtrate was collected and concentrated in rota evaporator at 40ºC. The extract was stored in refrigerator for further studies.leaves of Phyllanthus niruri were collected freshly from surroundings of SPMVV, Tirupati. The leaves were washed with clean tap water and then rinsed with distilled water to eliminate any dust on the surface. The adequately washed and cleaned leaves were dried at room temperature on clean tissue paper followed by blending. The powder was stored in an airtight container for further processing.

Preliminary Phytochemical screening

Preliminary phytochemical screening was performed by using standard procedure to test the presence of alkaloids, steroids, phenols, flavonoids etc [10].

Synthesis of Zinc oxide nanoparticles:

The green Synthesis of zinc oxide nanoparticles was carried out by adding 2% solution of aqueous extract to 100 ml of zinc acetate (0.01 M) with continuous stirring. The pH of the reaction system was adjusted to 12 with 2M NaOH by continuous agitation. The Agitation was continued until white precipitate has been formed [11]. After 15 min of Ultrasonication, the solution was subjected to centrifugation at 3000g for 15minutes in order to collect the pellet. The pellet was calcinated at 400˚C in Muffle furnace for 3 h.

Characterization of Zinc oxide nanoparticles

The synthesized nanoparticles was characterized by Ultra violet–Visible Spectrometer (Shimadzu UV-1800) ranging from 200nm to 600nm. The size and stability of the nanoparticles was determined by using DLS (HORIBA SZ-100).The presence of various functional group and reducing groups associated in the nanoparticles formation was assessed by Fourier transforms infrared spectroscopy (BRUKER / ALPHA-T) in the range of 400 cm^{-1} to 4000 cm^{-1}. In advance, the morphology and aggregation of nanoparticles was depicted by Scanning electron microscopy using Nano SEM 450 analyzer with acceleration voltageand the surface topology was captured with Atomic force microscopy (A100-AFM) 10 KV. The crystalline appearance of nanoparticles was characterized by X-Ray diffraction.

Antimicrobial activity

Agar disc-diffusion method was performed to determine the antimicrobial activity of ZnO nanopartcles [12] Nutrient agar plates. The test organisms (Escherichia coli (Gram -ve) Staphylococcus aureus (Gram +ve), Shigella (Gram -ve), Pseudomonas aeruginosa (Gram -ve), Aspergillus Niger, Candida albicans were cultured in nutrient broth to adjust the final inoculum of 1.5 × 108 CFU/mL. The nutrient agar plates were spread with above said inoculum. Sterile discs of 6 mm size(5no) was placed on cultured Agar plates. A ZnONs of 25µg, 50µg, 75 µg, 100µg concentration was added on discs with a positive control (Gentamycin 50µg) and negative control water in each experimental test organisms. The plates were allowed to prolix with nanoparticles for about 15 minutes at room temperature and incubated at 37°C, for 18-24 hrs (Different concentrations of ZnO nanoparticle such as 25µg, 50µg, 75µg, 100µg . After incubation plates were observed to assess the antimicrobial activity observed

DPPH free radical assay:

Free radical scavenging potential of ZnONP was assessed by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay[13]. Equal volume of 0.1 mM DPPH of ZnO-NPs (100, 200, 300, 400 and 500 µg) was incubated in the dark for 30 min. After incubation the scavenging potential was read at 517 nm by using ascorbic acid as standard and DPPH as control .

$$\% \text{ Scavenging activity} = \frac{\text{absorbance of Control- Absorbance of Sample}}{\text{Absorbance of Control}} \text{ X } 100$$

Preparation of starch based biofilm incorporated with Zno nanoparticles:

A solution of 4% starch was made by dissolving starch in lukewarm distilled water and adding 30% w/w glycerol. The addition of glycerol improved flexibility and decreased brittleness. 500 rpm was used to stir the solution for an hour. 3% of ZnO nanoparticles were carefully added. The pH of the solution was maintained by adding 5% acetic acid solution drop wise[14]. The mixture of starch, glycerol and ZnONPs was agitated at 80°C for 50–60 minutes until it became gelatinous. For control, starch was utilized. A Petri plate containing 20 milliliters of the film-forming solution was dried for 10 hours at 30°C in the oven. The prepared Biofilm with glycerol and starch was designated as (S+G). Biofilm prepared with 3% ZnONP, glycerol and starch was named as (S+G+ZnONP).

Antibacterial activity of biofilm

Antimicrobial activity of S+G biofilm and S+G+ZnONP was determined by Agar disc diffusion method. The test organisms such as (Escherichia coli (Gram -ve) Staphylococcus aureus (Gram +ve), Shigella (Gram -ve), Pseudomonas aeruginosa (Gram -ve) were inoculated in 50 ml of sterilized nutrient agar medium and poured on to petri dishes. A 10 mm S+G biofilm and S+G+ZnONP biofilm was placed over a top of the medium and all agar plates were incubated for 24hrs at 37°C. Finally, the zone of inhibition was calculated[14].

Cell lines maintenance

Vero cells (African green monkey kidney normal epithelial cell line) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator [15].

A study on Cytotoxicity effect

The cytotoxicity effect of S+G biofilm and S+G+ZnONP biofilm was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The Vero cell lines were seeded in 96-well microplates at a cell mass of 1 x 10^6 and the cells were incubated for 48 hours at 37°C in an incubator with 5% CO_2 until they reached 70–80% confluence. The cells are washed with phosphate-buffer saline (PBS, pH-7.4) and treated with MTT solution (5 mg/mL in PBS). The plates were incubated at 37ºC in the dark for 2h. The formazan crystals were dissolved in DMSO and the absorbance was read at 570 nm in Spectrophotometer [16]. Percentage of cell viability was calculated using the following formula.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \text{ X } 100$$

Bio film in food packaging

The selected fruit Strawberry and the vegetable Ivy gourd was collected from market, sterilized by washing with water and 70% ethanol. After washing allowed them to dry in sterile conditions. Manually the nanoparticles biofilm was wrapped around the selected fruit and vegetable [17]. Then they were stored for 3 days and then checked for the biofilm activity on food packaging.

Results and discussion

Preliminary phytochemical screening

The aqueous extract of Phyllanthus niruri was screened for phytochemical analysis in invitro conditions. Table.1 showed the list of levels of identified bioactive compounds, including phenols, flavonoids, alkaloids, Tannins etc. Among all screened phytochemicals, the flavonoids, coumarins, steroids, terpenoids are in high level than tannins and carbohydrates. These metabolites lend importantly to the extract's metabolic activity and shows powerful reduction mechanism and stabilizing capacities [18].

Table 1. Phytochemical composition of aqueous extract of Phyllanthus niruri

S.No	Phytochemical compounds	Aqueous extracts
1.	Flavonoids	++
2	Alkaloids	+
3.	Tannins	+
4.	Carbohydrates	+
5.	Coumarins	++
6.	Polyphenols	+++
7.	Steroids	+++
8.	Terpenoids	+++

Synthesis and characterization of ZnO nanoparticles

Phyllanthus niruri aqueous extract meditated ZnoNPs was formed by the redox reaction occurs between bioactive compound and zinc acetate under alkaline pH results in the formation of ZnONPs as a white precipitate (Figure-1). The formation of white colour is the indication of zinc acetate reduction by Phyllanthus niruri's chemical constituents, which act as an reducing agents, is reasoned to be a primary indicator.

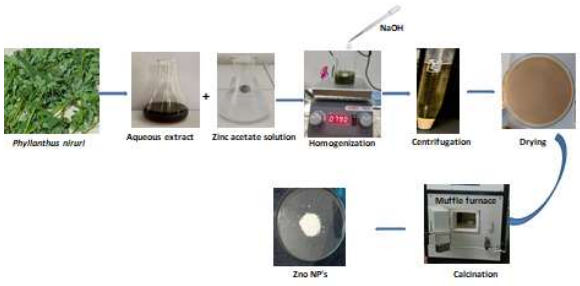

Figure.1. Green synthesis of ZnONPs using Phyllanthus niruri

Functional characteristics of ZnONPs' derived from Phyllanthus niruri

The distinct UV peak at 310 nm was confirmed the ZnONPs formation with a band gap energy 330eV. UV spectroscopy analysis display about the optical activity and materialistic behaviour of ZnONPs, which shows a band gap 330eV and absorption peak at 310nm. The results are associated with previous observations, from the ZnONPs which shows peak at 340nm [19] synthesized using Phyllanthis niruri whole plant extract. The FTIR spectrum of nanoparticles indicated that the functional groups of Phyllanthus niruri which involved in the reducing and capping stability of ZnONPs (Fig.2a). FTIR data was used to evaluate the responsible reactive groups involved in the reduction and stabilization of ZnONPS. The overwhelming broad spectrum of FTIR peaks of phyllanthus niruri's aqueous extract is 3400 cm-1 (Fig.2b) in ZnONPS the spectrum shows at 3451cm-1 represent the O-H stretching and the band shifted from 3400 cm-1 to 3451cm-1 indicating the interaction between O-H group and ZnO. The Molecular signature peaks observed at 1650 cm-1 1641 cm-1 and 1047.70 cm-1 correlated with the N-H bending and symmetric and asymmetric vibration of C=O and C–O stretching respectively [20]. The band observed at 1590.61cm-1 corresponds to the carbonyl group of flavonoids. The molecular signature at 489 cm-1 reflects the formation of Zinc oxide nanoparticles (Fig 2c).

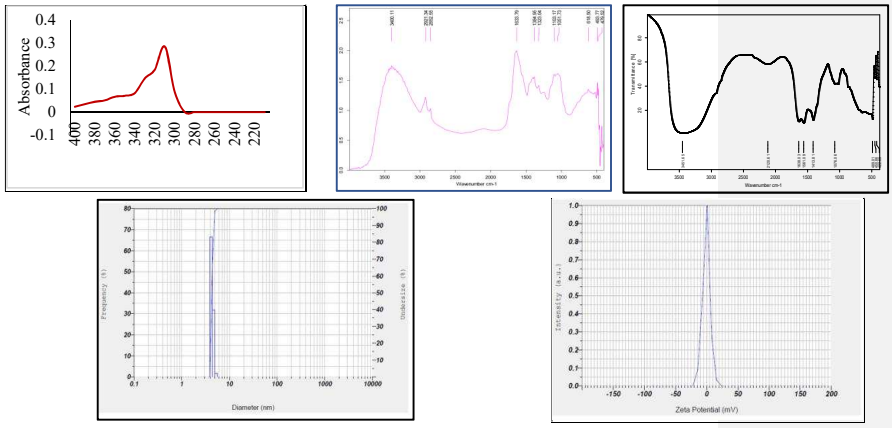

Figure. 2. Functional properties of ZnONPs synthesized using phyllanthus niruri

(a) Absorption maxima of Hb-ZnONPs (b) FTIR spectra of Aqueous extract (c)

FTIR spectra of ZnONPs (d) Particle size of Hb-ZnONPs (e) Zeta potential of Hb-ZnONPs (f) X-Ray diffraction pattern of Hb-ZnONPs

Dynamic light scattering used to discovered the mean size of ZnONPs as 9.5 nm (Fig. 2d), and in advance the zeta potential is a significant parameter for determine the dispersion stability and electrostatic potential of ZnONPs and it was found to be -44.7mV (Fig. 2d). The order of magnitude of zeta potential smaller than -50mV and grated +50mV form stable suspension and reduce aggregation. The above said techniques have been used to know the nanoparticle identification and hypothesized their extreme properties including shape, size , charge, elemental composition[21].

Morphological changes of ZnONPS

SEM, XRD results strengthen the fabrication of Crystalline and hexagonal appearance of nanoparticles with average size of 9.5nm was confirmed through SEM, XRD results. This structural characteristics making them more significant for different applications [22]. The synthesized nanoparticles showed a hexagonal properties. (Figure.3). The seze of the nanoparticeles in SEM revealed the size range from 40nm to 75nm. The peaks corresponding to carbon, sodium or its OH compounds were not observed in the XRD pattern, which indicates the absence of additional phases in the bio-synthesized nanoparticles.

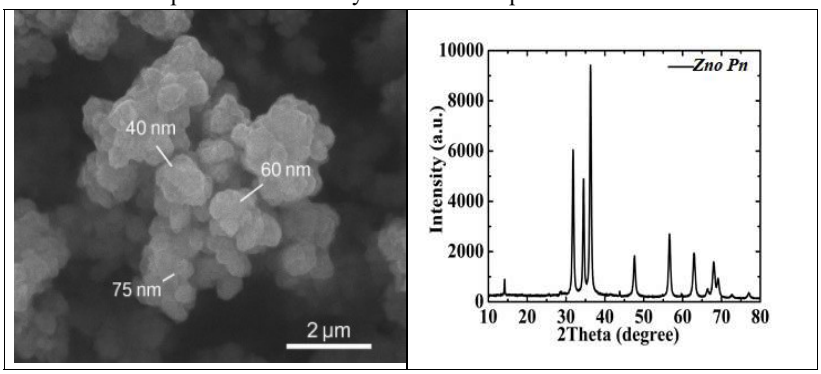

Figure. 3 Structural features of ZnONPs synthesized using Phyllanthus niruri

The sharp and narrow diffraction peaks in the XRD spectrum indicate that the ZnO nanoparticles are **highly crystalline**. The absence of any extra peaks suggests **phase purity** and **successful synthesis without contamination** from other metal oxides or precursors. The crystalline size estimated from the XRD analysis is consistent with typical values reported for ZnO nanoparticles synthesized by methods such as sol-gel, precipitation, or green synthesis [23]. The surface of the ZnO nanoparticles appeared **rough and porous** under high magnification, indicating a **high surface area**, which is beneficial for applications requiring surface interaction—such as **antibacterial activity in food packaging**.

Effect of Phyllanthus niruri ZnONPs **and nano biofilm on proliferation of Verocell lines**

The aqueous extract of Phyllanthus niruri at all concentration shows the intact shape and morphology and it stands for non toxic nature along with ZnONP. The cell proliferation percentage of cells was compared with control cells, the cell viability dropped to 78 % at 500 µg and 90% at 100 µg of ZnO nanoparticles. In contrary, the aqueous extract showed non toxicity in cell lines and the IC_{50} values for aqueous extract and nanoparticles was found to be

320µg. The toxicity at higher concentration of ZnONPs shows the disequilibrium between zinc mediated cellular activity and redox responses [24]. Production of ROS can be the primary reason as it disrupts mitochondrial function leading to cytotoxicity. ZnONPs showed significantly less cytotoxicity. Due to low toxicity, the nanoparticles were used for production of biofilm for active food packaging.

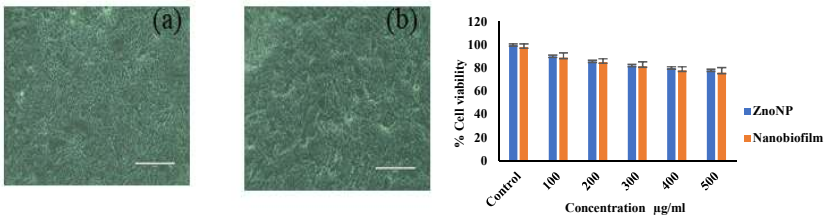

Figure.4. Microimagess Veero cell proliferation (a) Proliferation of Vero cells in the presence of phyllanthus niruri extract (b) Proliferation of Vero cells in the presence of ZnONPs

Antimicrobial activity of ZnONPs and Nanobiofilm

The zone of inhibition of bacterial growth was shown in Table.1. Green synthesized ZnO nanoparticles shows highest antimicrobial activity at 100µg/ml concentration on all test organisms. The highest zone of inhibition (21±1.02) showed at 100µg/ml against E.coli. Followed by Shigella, S. aureus , P. aeruginosa, C. albicans, A. niger .The antimicrobial effectivity of green synthesized P. niruri ZnONps may be due to the presence of active compounds in P. niruri leaf extract which act as capping agent in Nps and to reduce the size of particles and enhances the antimicrobial activities [25]. When nanoparticles are small in size increasing the antimicrobial activity, this is due to the small size particle having higher surface to volume ratio, so smaller size may increase the penetration with in bacterial cell membrane and causes bactericidal effect [26]. Based on the XRD patterns, it is defined that the crystalline size also lead to the biological activity of ZnONPS. The antibacterial activity of nano-biofilm was also evaluated. The biofilm showed significant antimicrobial activity (Figure. 5).

Table.2 Antibacterial Activity of green synthesized ZnONpsws

Diameter of zone of inhibition in mm						
Concentration of nanoparticle	**Shigella**	**E.coli**	**S. aureus**	**P. aeruginosa**	**C. albicans**	**A. niger**
25	5±1.22	14±1.12	6±1.42	8±1.02	4.0±0.02	3.0±0.32
50	15±1.43	16±1.20	15±1.52	14±1.16	8.6±1.32	7.5±1.09
75	16±1.51	19±0.67	16±0.32	16±1.09	18±1.43	15±1.8
100	18±1.4	21±1.02	19±1.11	17±1.32	20±1.82	18±1.4
Gentamycin	21±0.32	22±1.09	21±1.02	23±0.82	24±0.72	20±1.7

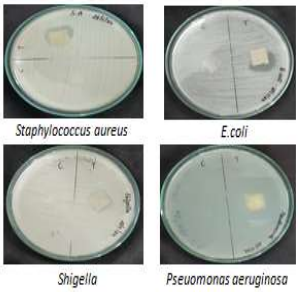

Figure.5 Antibacterial Activity of green synthesized ZnONPs Biofilm

Zno nanoparticles have broad spectrum activity against various bacteria and fungi. ZnO nanoparticles exhibited the **promising antimicrobial activity** against a wide range of food borne bacteria and fungi. Their integration into food packaging materials can enhance **food safety, reduce microbial spoilage, and extend product shelf life**.

Free radical scavenging activity

According to the findings, the food packaging biofilm prepared with ZnONPs showed significant dose-dependent scavenges DPPH radicals. The inclusion of phenolic chemicals, flavonoids, or essential oils included in the ZnONPS packaging material's is probably gives its antioxidant ability.

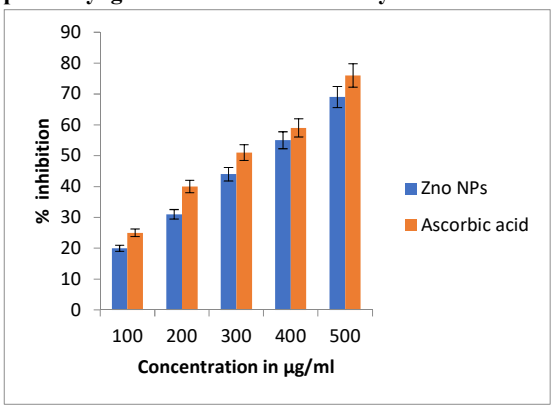

Figure.6. Free radical scavenging activity of ZnO nanoparticle

Strategies for incorporating zno-nps in food packaging

ZnO nanoparticles' exceptional qualities, including their antibacterial, UV-blocking, and antioxidant actions, have drawn a lot of interest from the food packaging sector [27]. By inhibiting bacterial development and reducing damaging UV radiation, these nanoparticles can help extend the shelf life and safety of food goods.They are a perfect fit to be used in food packaging materials because of their compact size and large surface area [28]. ZnO nanoparticles can be included into food packaging using a variety of techniques, including as coating, embedding in nanocomposites, and blending with polymers . These methods present

a viable remedy. to satisfy the rising need for environmentally friendly and useful food packaging materials. This review will go over a few of these techniques and uses [29].

Biofilm used in Active food packaging

The efficacy of ZnO nanoparticle (ZnO NP)-embedded biofilm as an antimicrobial food packaging material was evaluated by wrapping perishable fruits such as straw berries and Ivy guard [30]. The aim is to assess the film's **preservation capability**, including **microbial load reduction, visual quality retention**, and **extension of shelf life** during ambient storage conditions. The results confirm that ZnO nanoparticle-embedded biofilms are effective active packaging material with antimicrobial action [31]. Compared to control films, the ZnO NP film performed better in reducing microbial contamination, preventing spoilage, and extending shelf life under non-refrigerated conditions [32]. These findings are particularly relevant for perishable produce storage in rural and non-refrigerated settings, where maintaining food safety and quality is challenging [33]. The activity of biofilm active packing has been shown in Figure.6 &7

Figure 7: Fruits wrapped with control film and nanoparticle incorporated biofilm – Day 1

Figure 8: Fruits wrapped with control film and nanoparticle incorporated biofilm – Day 2

Conclusion

This describes the use of the bio-reduction approach to synthesize ZnO NPs. Numerous polyphenols, flavonoids, sugars, and proteins found in the leaf extract of the phyllanthus niruri plant function as both stabilizing and reducing agents during the synthesis of ZnO NPs. The production of ZnO was confirmed by the UV absorption peak. Nanopartickes at 310 nm, and XRD and SEM analyses confirmed the hexagonal structure, which has a size range of 9.5 nm. The findings of the anti-biofilm testing indicated that, across the range of doses tested, the

synthesized ZnO NPs exhibited a significant degree of biofilm detachment characteristic. The significant antibacterial activity of the produced ZnO NPs was confirmed by the fact that neither Gram-positive nor Gram-negative bacteria could multiply in the presence of the nanoparticles.

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Authorship contribution

All the authors contributed extensively to the work presented in this manuscript.

Conflict of interest : The author(s) report no conflicts of interest in this work

Data availability: The datasets generated and/or analyzed during the current study are available in the manuscript

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

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