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ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF PHYTOSYNTHESISED ZnOs BY *Hibiscus tiliaceus* LEAF EXTRACT AGAINST FOUR PATHOGENIC BACTERIA

O.K. Putri[1,2], H. Syafdhani[1], Holilah[1], A. Fadlan[1], Y. Kusumawati[1,✉], M. Santoso[1], D. Prasetyoko[1], and H.J. Achmad[1]

[1]Department of Chemistry, Faculty of Science and Data Analytics, Sepuluh Nopember Institute of Technology (ITS), Surabaya, Indonesia, 60111.

[2]Department of Pharmacy, Academy of Pharmacy of Putra Indonesia Malang, Malang, Indonesia, 65122.

✉Corresponding Author: y_kusumawati@chem.its.ac.id

ABSTRACT

The numerous benefits provided by Zinc oxide have attracted attention. Therefore, plenty of ZnO synthesis methods have been developed. This study reported the phytosynthesis of ZnOs by *Hibiscus tiliaceus* leaf extract, the characterization of phytosynthesised ZnOs, and their antibacterial and antioxidant activities. The *H. tiliaceus* leaf extract was prepared with distilled water and distilled water/ethanol mixtures at three different ratios. The characteristics of the phytosynthesised ZnOs were revealed by infrared spectroscopy, scanning electron microscopy, and X-ray diffraction. A spectrophotometric technique was enforced to investigate the antioxidant activity of phytosynthesised ZnOs and the total phenolic level of the extracts. The antibacterial activity was assayed against four pathogenic bacteria. The particle sizes of the phytosynthesised ZnOs were 46 nm to 273 nm with rod and hexagonal shapes. The highest DPPH free radical scavenging activity was shown by phytosynthesised ZnOs produced from extract with a ratio of aqua dest: ethanol, which was 1:1 (v/v). Strong antibacterial activity toward *Staphylococcus epidermidis* was displayed by phytosynthesised ZnOs compared to *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*. The phenolic and flavonoid compounds of *H. tiliaceus* leaf extract affected the antioxidant activity of phytosynthesised ZnO. The inhibition zones of some phytosynthesised ZnOs were better than pharmaceutical-grade ZnO.

Keywords: Antibacterial; Antioxidant; *Hibiscus tiliaceus*; Phytosynthesis; ZnO Nanoparticles.

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INTRODUCTION

The *Hibiscus* genus commonly found in Southeast Asia has a member known as *Hibiscus tiliaceus*. *H. tiliaceus* is a small coastal plant and is known as waru, Baru, or haru in Indonesia. The leaves and bark of this plant are widely used in traditional medicine as a result of important natural active compounds exist.[1] The secondary metabolites contained include anthocyanins, coumarins, phenolic acids, phytosterols, triterpenoids,[2] and saponins.[3] *H. tiliaceus* provides pharmacological effects, such as analgesic, antiallergy, antibacterial, anticancer, antidiabetic, anti-inflammatory, antioxidant, antiviral, filaricidal, and hepatoprotective activities.[2] The leaves of *H. tiliaceus* have been noted to be rich in phenolic and flavonoid compounds compared to bark, fruits, and roots. Several phenolic compounds, namely, catechins, ellagic acid, quercetin, and rutin hydrate, have been isolated from the ethanol extract of *H. tiliaceus* leaves.[4]

Metal oxides can be applied to various fields, especially industrial and consumer products, because of their unique properties.[5] Zinc oxide is a metal oxide with the most extensive applications in the fields of health care, pharmaceuticals, environmental remediation, food additives, and industries, making this material interesting to study. ZnO has various medical applications, such as antidiabetic,[6] antimicrobial, anti-inflammatory, antioxidant, anticancer,[7] antifungal,[8] thyroid disease cures,[9] antidermatophytic,[10] antianxiety, pulmonary, and hepatoprotective activities.[7] Studies have also shown that ZnO can be used in wastewater treatment, including hospital wastewater and environmental remediation of harmful pollutants.[11,12] Therefore, in addition to being beneficial to health, ZnO also has benefits for environmental issues. Chemical, physical, and biological techniques can be utilized to fabricate metal oxides. Among

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others, biological methods are commonly referred to as "green" techniques due to their ability to produce metal oxides with good resistance and biocompatibility.[13] Biological methods also do not require expensive chemicals, use lower energy, lower pressure, and produce environmentally friendly byproducts.[11] Biological methods involve microorganisms (unicellular and multicellular), plants, seaweeds, viruses, bacteria, or their by-outputs, such as lipids and proteins, for synthesizing metal oxides. Plants are the most widely used media in the preparation of metal oxides compared to others, and this technique is called photosynthesis. This is because plants offer simple and straight preparations, are nonpathogenic and economical, and produce more stable products with more morphological variations.[14] Plants are also known for their phenolic,[11] and flavonoid[15] compounds which play important roles in the formation of metal oxides. Studies show that flavonoids in leaf extracts can increase the bioactivity and bioavailability of metal oxides. For example, ZnO phytosynthesized using aloe extract had higher antibacterial activity than ZnO synthesized by chemical methods.[16] This study, therefore, aimed to prepare ZnO by photosynthesis using *H. tiliaceus* leaves, characterize phytosynthesized ZnOs, and investigate their antibacterial and antioxidant activities. The phenolic and flavonoid compounds in *H. tiliaceus* leaves have potential as photosynthetic agents[17] for ZnO.

EXPERIMENTAL

Materials

H. tiliaceus leaves were obtained from Sidoarjo, East Java, Indonesia. Zinc nitrate [$Zn(NO_3)_2{\cdot}6H_2O$] (Merck), ethanol (Merck), methanol (Merck), gallic acid (Merck), SS agar (Merck), CM0069 eosin methylene blue agar (Oxoid), nutrient agar (Himedia), ZnO pharmaceutical-grade (locally manufactured) (Brataco), Folin-Ciocalteu reagent (Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich), and sodium carbonate (Merck) were used as received.

H. tiliaceus Leaf Preparation and Extraction

Before grinding, *H. tiliaceus* leaves were washed with tap water, a mixture of ethanol and water (50% v/v), and distilled water successively. *H. tiliaceus* leaves were then dried at 60 °C for one day and ground. A total of 10 g of each *H. tiliaceus* powder was poured into different solvents at a volume of 100 mL: distilled water, a mixture of distilled water, and ethanol at ratios of 3:1, 1:1, and 1:3 (v/v). To obtain the extracts, the mixtures were stirred at 70 °C for 20 min, precipitated at 6000 rpm for 10 min by centrifugation, and filtered.

Total Phenolic Content (TPC) Determination of *H. tiliaceus* Leaf Extract

The Folin-Ciocalteu technique with gallic acid as a standard solution was utilized to determine the TPC of *H. tiliaceus* leaf extract.[18] A standard curve of gallic acid was constructed after obtaining the maximum wavelength of the gallic acid solution. The samples were provided by homogenizing 0.01 mL of each extract with 0.5 mL of methanol, 2.5 mL of distilled water, and 2.5 mL of 50% Folin-Ciocalteu reagent followed by incubation for 5 minutes. After that, 2 mL of 7.5% Na_2CO_3 solution was poured, and the mixture was further incubated for 15 min. Next, the absorbance of the samples was measured at the maximum wavelength. The absorbance of the samples was plotted to the standard curve equation of gallic acid to obtain the TPC and reported as gallic acid equivalent (GAE) per gram of sample.

Preparation of Phytosynthesized ZnOs

Preparation of phytosynthesized ZnOs was carried out by a 5-g aliquot of $Zn(NO_3)_2{\cdot}6H_2O$ was mixed with 30 mL of *H. tiliaceus* leaf extract, and then stirred at 85 °C for 20 h. The collected slurries were heated at 400°C for 2 h.

Characterization of Phytosynthesized ZnOs

To specify the crystal structure and crystallinity of ZnOs, an X-ray diffractometer (PHILIPS-binary X'Pert MPD) with Cu Kα (1.54 Å), 2θ at 5-90°, and a scanning interval of 0.017° were used. The functional groups of phytosynthesized ZnOs were detected by using a Fourier transform infrared (FTIR) spectrometer (SHIMADZU 8400S) within the interval of 4000-400 cm^{-1}. Scanning electron microscopy (JEOL JSM-6390) with Au-Pd coating samples was used to evaluate the surface morphology and distribution of ZnOs.

Antioxidant Activity Determination

Phytosynthesis ZnOs at various concentrations (3 µg/mL, 6 µg/mL, 9 µg/mL, and 12 µg/mL) were antioxidant activity assayed by adding to 4 mL of methanol and 1 mL of DPPH 500 µg/mL solution in methanol. The mixture was shaken and then located for 30 minutes in the dark at room temperature. A UV-Vis spectrophotometer (HITACHI U-2900) was utilized to check the sample's absorbance at 517 nm wavelength. The sample antioxidant characteristic was determined by calculating the IC_{50} (the half maximal inhibitory concentration) value.

Antibacterial Activity Determination

The agar well diffusion was used to figure out the ZnO's antibacterial properties against several pathogenic bacteria, including gram-positive (*S. aureus* and *S. epidermis*) and gram-negative (*E. coli* and *S. Typhi*). *S. epidermidis* was obtained from the University of Brawijaya, Indonesia, whereas *S. aureus, E. coli*, and *S. Typhi* were purchased from the University of Muhammadiyah Malang Indonesia. The rejuvenated bacteria were suspended in a physiological NaCl solution (0.9% NaCl), and the absorbance at 25% T was measured on a UV-Vis spectrophotometer (Thermo Fisher Scientific Genesys 10S UV-Vis) at a wavelength of 580 nm for *S. aureus*, *S. epidermidis,* and *S. Typhi* and at 620 nm for *E. coli*. A 1mL bacterial suspension was poured into a Petri dish, added to nutrient agar media, and solidified. A sterile metal tube was punched on the agar plates, and each of the tubes was filled with ZnO suspension at concentrations of 500, 1000, 2000, and 5000 µg/mL and placed in an incubator at 37 °C for 24 h. The ZnO suspensions were made by mixing ZnO powder with distilled water and then homogenized with 42 kHz ultrasonic waves for 30 min followed by sterilization by an autoclave.[19] The ZnO suspension was homogenized by vortexing before being poured into the well.

RESULTS AND DISCUSSION

TPC of *H. tiliaceus* Leaf Extract

The extraction indicated that the greater the amount of ethanol in the extraction solvent, the more concentrated the extract. TPC of *H. tiliaceus* leaf extracts from distilled water (a), distilled water: ethanol mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d), as shown in Fig.-1 indicated that the distilled water: ethanol (1:1) extract had the highest TPC (10.87±0.03 mg GAE/mL). A previous study reported that single solvent systems are less efficient than binary solvent systems in extracting polyphenolic compounds due to their relative polarity.[20] The ratio of ethanol in the solvent mixture was very important so that the phenolic compounds could be extracted efficiently. A study showed that 1 g of *Actinidia arguta* leaf powder extracted with 50 mL of the same solvent under constant stirring at 50 °C for 1 h gave a TPC of 140.72±10.22 mgGAE/g.[21] Although the extraction was realized applying the same method and solvent, the differences in the ratio of leaf powder and solvent, temperature, and duration of extraction caused different total phenolic levels. The type and concentration of solvent, also duration and temperature of extraction affect the TPC.[22]

Phytosynthesized ZnOs and Characterization

$Zn(NO_3)_2 \cdot 6H_2O$ acts as a source of Zn^{2+} ions, and *H. tiliaceus* leaf extract operates as a ZnO stabilizer and reducing agent in the preparation of phytosynthesised ZnOs. The extraction solvents for the extracts were distilled water and distilled water and ethanol mixtures at ratios of 3:1, 1:1, and 1:3 (v/v). The masses of ZnO obtained from phytosynthesis with those extracts were 0.572 g, 0.631 g, 0.701 g, and 0.611 g, respectively. All phytosynthesised ZnOs were produced as white powders and were insoluble in water and dimethyl sulfoxide (DMSO). This finding was in accordance with previously reported papers.[23] The XRD pattern of phytosynthesized ZnOs from distilled water (a), distilled water: ethanol mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) extracts are presented in Fig.-2. The obtained diffractograms were fitted with JCPDS No. 36-1451 from the International Center of Diffraction Database 2001 for ZnO by using the PCPDFWIN program. The standard has typical characteristic peaks at 2θ = 31.770°, 34.422°, and 36.253°. The diffractograms of four phytosynthesised ZnO powders agreed with this standard. The four XRD diffraction patterns showed sharp peaks, which meant that the phytosynthesised ZnOs were highly crystalline. The presence of various peaks that are identical to the standard Miller index indicates that the phytosynthesis of ZnO formed has a hexagonal structure.[24] No additional peaks were found except those previously in the pattern of XRD, indicating that ZnO was highly pure.

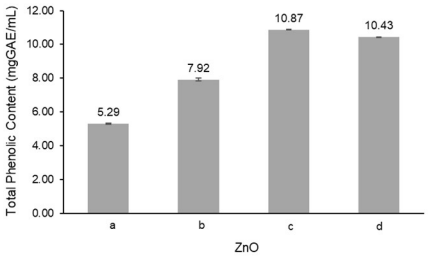

Fig.-1: TPC of *H. tiliaceus* Leaf Extracts From Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d)

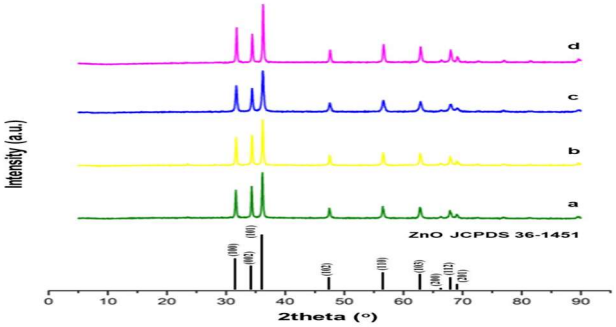

Fig.-2: XRD Diffractograms of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) Extracts

FTIR study was enforced to figure out the functional groups contained in phytosynthesized ZnOs. The FTIR results of phytosynthesized ZnOs from distilled water (a), distilled water: ethanol mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) extracts, as shown in Fig.-3 exhibited peaks in the range of 400-500 cm^{-1}, which correspond to the stretching vibrations of the Zn-O band.[25] The bands found at 3400-3500 cm^{-1} can be caused by O-H vibrations. Another supporting fact that strengthens the presence of O-H is the presence of high-intensity bands at 1300-1400 cm^{-1}, which is identical to the O-H in-plane bending vibration. These results indicate that there are aqua, hydroxy, and/or oxo species on the surface of the material so that it provides excess oxygen content and is identified in the infrared spectra.[26] The low-intensity band at 1000-1100 cm^{-1} confirmed the C-N stretching of aliphatic amines. The amine groups in the *H. tiliaceus* leaf extract play an important role as a source of natural bases for the production of $Zn(OH)_2$ to become ZnO in the calcination process.[27]

The morphology, size, shape, and arrangement of phytosynthesized ZnOs were analyzed by using scanning electron microscopy. Micrographs of phytosynthesized ZnOs from distilled water: ethanol mixtures 3:1 at 50000 (a) and 100000 (b) times magnification, distilled water: ethanol mixtures 1:1 at 50000 (c) and 100000 (d) times' magnification are displayed in Fig.-4. Fig.-4a and 4b show mixed rod-shaped and hexagonal-shaped with particle sizes between 62 nm to 273 nm. The hexagonal-shaped particles support the results obtained from X-ray diffraction.

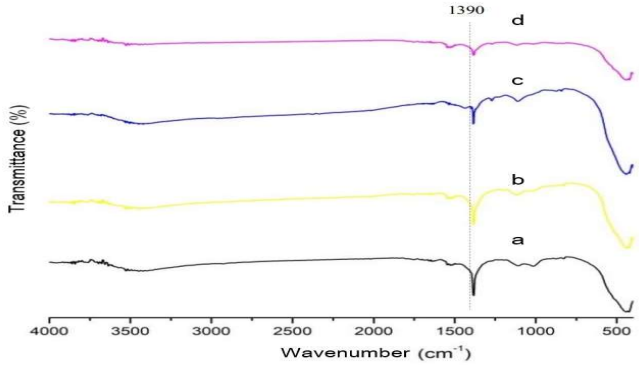

Fig.-3: FTIR Spectra of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) Extracts

While Fig.-4c and 4d show smaller particle sizes between 46 nm to 125 nm. It is known that extracts with higher ethanol solvents favor the complexation of Zn(II) and produce highly identical particles.[28] Particles with a diameter of <1,000 nm can be accepted as Nano sized carriers that can be used in the pharmaceutical industry.[29] Different morphologies of ZnO can be produced from biosynthesis using various plant extracts. ZnO nanoparticles synthesized from extracts of various types of fruit peels and zinc nitrate precursors have a polyhedral shape.[30] ZnO nanoparticles produced from *H. sabdariffa* leaf extract combined with zinc acetate precursor are spherical in shape and form agglomerates.[31] Irregularly shaped ZnO was produced using *Phyllanthus niruri* leaf extract and zinc nitrate precursor.[32]

Antioxidant Activity

The prevalent antioxidant method, the DPPH assay used to perform the antioxidant evaluation of phytosynthesized ZnOs.[33] The DPPH free radical scavenging technique is related to the reduction of colored DPPH by free radical inhibitors. The activity is expressed as the 50% inhibitory concentration or IC_{50}. The lower the IC_{50} value is, the stronger the antioxidant activity of a substance. Several studies have explained the ability of polyphenols to produce significant antioxidant properties.[34]

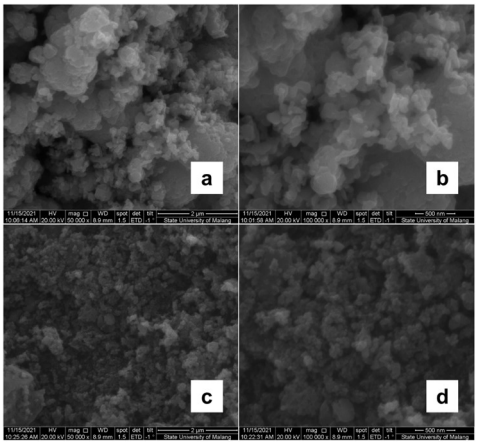

Fig.-4: SEM Micrograph of Phytosynthesized ZnOs from Distilled Water: Ethanol Mixtures 3:1 at 50000 (a) and 100000 (b) Times Magnification, Distilled Water: Ethanol Mixtures 1:1 at 50000 (c) and 100000 (d) Times Magnification

Antioxidant activity of phytosynthesized ZnOs from distilled water (a), distilled water: ethanol mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) extracts as shown in Fig.-5 shows that the highest free radical scavenging ability was shown by phytosynthesised ZnO using distilled water: ethanol extract at a ratio of 1:1 with an IC_{50} amount of 21.55±0.08 mg/mL. This result was supported by the high TPC of the same extract. This was also confirmed by the peak at the wavelength of 1390 cm^{-1} in the infrared spectra, indicating C-O-H vibrations related to the functional groups in phenolic compounds. The IC_{50} value closest to this study's outcomes is indicated by the biosynthesized ZnO from the fruit extract of *Artocarpus gomezianus* (10.8 mg/mL).[35]

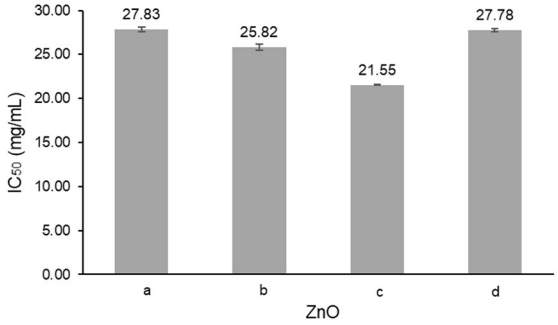

Fig.-5: Antioxidant Activity of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), and 1:3 (v/v) (d) Extracts

Antibacterial Activity

Several pathogenic bacteria, including gram-positive *S. aureus* and *S. epidermidis* and gram-negative *E. coli* and *S. Typhi* were utilized to confirm the antibacterial activity of phytosynthesised ZnOs. The assay was conducted by agar well diffusion technique. Figure-6 to 9 displayed the results for phytosynthesised ZnOs from distilled water (a), distilled water: ethanol mixtures 3:1 (b), 1:1 (c), 1:3 (v/v) (d), and pharmaceutical-grade ZnO (e). Figure-6, Fig.-7, Fig.-8, and Fig.-9 show the antibacterial character of ZnOs against *S. aureus*, *S. epidermidis*, *E. coli*, and *S. typhi*, sequentially. The phytosynthesised ZnOs exhibited stronger antibacterial activity toward *S. epidermidis* at various concentrations (Fig.-7). The gram-positive bacterial cell wall is composed only of peptidoglycan and a single plasma membrane. Gram-negative bacteria are composed of an outer plasma membrane, an inner plasma membrane, and peptidoglycan. The outer plasma membrane in the cell wall can protect the bacteria by blocking the entry of antibiotics and the host defense system.[36] However, the exact mechanism of cell membrane damage is still under debate. In general, as shown in Fig.-6 to 9, the high concentration of phytosynthesised ZnOs was not always linear with the inhibition zones. Moreover, no inhibition zones were detected at several concentrations for *S. aureus* and *S. typhi*.

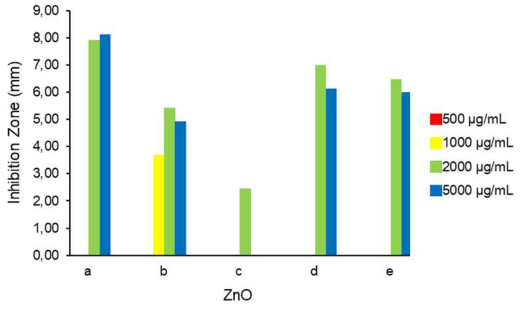

Fig.-6: Antibacterial Activity of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), 1:3 (v/v) (d), and Pharmaceutical-Grade ZnO (e) against *S. aureus*

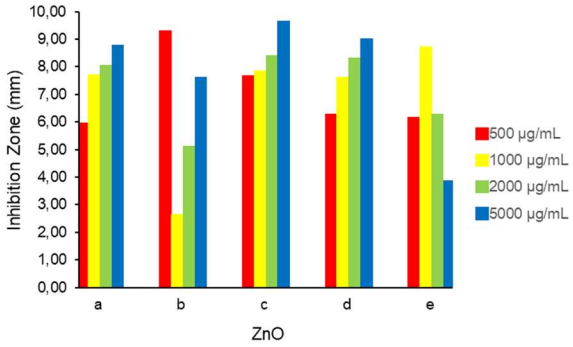

Fig.-7: Antibacterial Activity of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), 1:3 (v/v) (d), and Pharmaceutical-Grade ZnO (e) against *S. epidermidis*

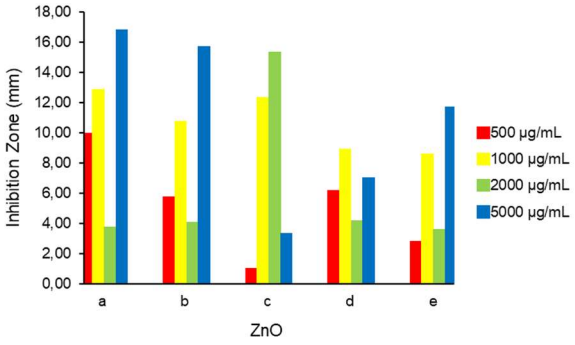

Fig.-8: Antibacterial Activity of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), 1:3 (v/v) (d), and Pharmaceutical-Grade ZnO (e) against *E. coli*

A reported paper explained that ZnO has shown significant antibacterial activity, but the exact mechanism of ZnO toxicity against bacteria is still unclear.[37] Thus, numerous factors, such as the effective size and morphology of ZnO and the bacterial species tested, could affect the antibacterial activity of the synthesized ZnO.[38] A similar trend of inhibition zones was shown by the phytosynthesized ZnOs with various extracts and pharmaceutical-grade ZnO against all tested bacteria. The maximum inhibition zone for phytosynthesized ZnO was 16.60 mm for *E. coli* and 8.04 mm for *S. aureus*. Both were obtained from phytosynthesized ZnO with distilled water: ethanol mixtures of 3:1 at a concentration of 5000 µg/mL. Meanwhile, the pharmaceutical-grade ZnO produced maximum inhibition zones of 11.49 mm for *E. coli* and 5.90 mm for *S. aureus* at the same concentration. Similar results were shown by ZnO (Sigma–Aldrich) and phytosynthesized ZnO from *A. vera*, which was 18.33 mm and 26.45 mm for *E. coli* and 22.11 mm and 28.12 mm for *S. aureus, respectively*.[39] Phytosynthesized ZnOs can inhibit *E. coli* and *S. aureus* bacteria better than commercial ZnO. The highest zone of inhibition against *S. epidermidis* with a value of 9.55 mm was indicated by phytosynthesised ZnO from 1:1 distilled water: ethanol mixtures at a concentration of 5000 µg/mL, which was larger than the ZnO pharmaceutical-grade (3.76 mm). Nonbiosynthetic ZnO with a particle size of 50 nm provided a 15-mm inhibition zone at the same concentration.[40] A previous antibacterial activity test showed that no activity was observed against *S. typhi* for phytosynthesized ZnO using *Berberis aristata* leaf extract.[41] In this study, a better inhibition zone was obtained, which was 24.02 mm from phytosynthesized ZnO from 1:3 distilled water: ethanol mixtures at a concentration of 2000 µg/mL. This result is also better than the results of pharmaceutical-grade ZnO with the same concentration, which gave a 19.01-mm inhibition zone. Phytosynthesised ZnO has strong potential as an antibacterial agent which has been confirmed by previous research.[42]

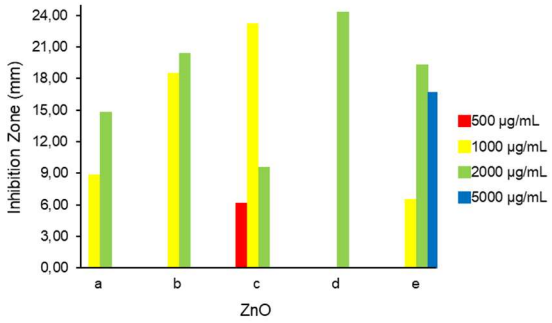

Fig.-9: Antibacterial Activity of Phytosynthesized ZnOs from Distilled Water (a), Distilled Water: Ethanol Mixtures 3:1 (b), 1:1 (c), 1:3 (v/v) (d), and Pharmaceutical-Grade ZnO (e) against *S. typhi*

CONCLUSION

ZnO has been successfully synthesized with the assistance of *Hibiscus tiliaceus* leaf extract in various solvents. X-ray diffraction and infrared spectroscopy have successfully confirmed the phytosynthesized ZnOs. The particle sizes of phytosynthesized ZnOs were 46 nm to 273 nm with rod and hexagonal shapes. The phytosynthesized ZnOs displayed antibacterial activity, and some ZnOs had better inhibiting zones than pharmaceutical-grade ZnO. The phytosynthesized ZnO produced from the extract with distilled water: ethanol (1:1) showed higher antioxidant activity than the others.

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