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Preparation, Characterization, Antioxidant and Antimicrobial Activities of Lignin and Eco-friendly Lignin Nanoparticles from Egyptian Cotton Stalks

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Abstract

Pathogenic microorganisms are a major source of environmental concerns, according to their capability of proliferation on all surfaces. Antimicrobial drugs of new types are now has been developed to inhibit bacteria adherence and biofilm formation. However, they are generally of synthetic origin and have a lot of disadvantages. Natural biopolymers as cellulose, hemicellulose, and lignin, look interesting as antibacterial agents. Lignin, a common plant biopolymer, is known to have antioxidant activity. Nanoparticles have qualities that are either better or worse than their bulk materials or parent polymers. The objectives of this article are to recycle cotton stalks into environmentally friendly products by extracting lignin from two Egyptian cotton cultivars (by-products): Giza 86 and Giza 90, using the organosolv technique, and transferring them into lignin nanoparticles. Also, to determine whether lignin and lignin nanoparticle bioactivities (i.e., antioxidant and antibacterial) may be used to treat textiles for medical applications against seven harmful bacteria (*Staphylococcus aureus, Staphylococcus sciuri, Bacillus cereus, Salmonella enterica, Salmonella typhi, Escherichia coli, and Pseudomonas aeruginosa*) and five mycotoxigenic fungi (*Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger, Fusarium proliferatum, and Penicillium verrucosum*). LNPs were studied using Particle Size Analyzer, Fourier Transformer Infrared Spectroscopy (FTIR), and transmission electron microscope (TEM). The results revealed that organosolv lignin nanoparticles 90 (OLNP90) had the strongest antioxidant effectiveness with an IC50 of 9.41 µg/mL whereas the minimum antioxidant activity for organosolv lignin nanoparticles 86 (OLNP86) was found with an IC50 of 11.66 µg/mL. On the other hand, treatment with lignin and lignin nanoparticles had a greater effect on bacteria than fungi. *Penicillium verrucosum* exhibited the greatest inhibition zone (15.5 mm) for OLNP86 with no significant differences with Nystatin (15.8 mm), while *Salmonella typhi* exhibited the greatest inhibition zone (14.7 mm) for OLNP86 in comparison of Tetracycline (25.4 mm). Similarly, the study of textiles treated with lignin and nano-lignin showed a clear effect on bacteria and fungi under study, where *Penicillium verrucosum* exhibited the greatest inhibition zone (30.7 mm), while *Pseudomonas aeruginosa* exhibited the greatest inhibition zone (36.3 mm) for OLNP86. As a result, the current study showed that OLNP's antioxidant and antimicrobial characteristics can be further utilized in medical textiles (surgical bandages, clothing, bed sheets, and towels), which can be used for patients with conditions such as diabetes, high blood pressure, and kidney failure who cannot tolerate antibiotics.

Key words: cotton stalk; organosolv lignin; lignin nanoparticles; TEM; antioxidants; antimicrobial; textile

1. Introduction

The cotton stalk is the main agricultural by-product of the cotton plant; it is postharvest trash left in the field after picking seed cotton [1]. Cotton stalk recycle possesses a lot of benefits, including environmental protection from GHG (greenhouse gases) and insecticides used to prevent pink bollworm (which were present during cotton stalk storage) [2]. Creating new jobs from uprooting to selling, instead of burning or disposing of it in a landfill, by turning it from worthless trash to high-value materials like lignin [2,3]. The cotton stalk account for 68,000,000 Bone Dry Metric Tons globally [4]. Also, it produced 4.5 tonnes/ha in Egypt

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in 2019/2020 [5]; hence the cotton stalk yield from 238095 feddans planted of cotton was 1071482 tonnes. The main constituents of cotton stalk are cellulose (69%), lignin (27%), and hemicellulose (14.4%)[3].

Lignin, the second most prevalent natural polymer, eco- cellulose and hemicellulose; it is made up of a propanoid chain and an aromatic ring with hydroxyl and methoxy functional groups, the solubility of lignin that has processed with organosolv has greatly improved [6]; The significant antioxidant activity of lignin is dependent on its solubility in the reaction system; Organosolv lignin has the highest free radical scavenging activity. Low antioxidant activity is caused by hydrogen bonding between phenolic hydroxyl groups of lignin and carbohydrates [7]. Lignin has always piqued researchers' curiosity as the second most widely distributed natural polymer after cellulose and a renewable resource. Lignin became a very intriguing topic for researchers and businesses as a result of this. However, because of its intricacy and unevenness, it has been impossible to determine its specific structure, as well as how it may be turned into a commonly used raw material, until now. Because of the significant changes in the native lignin structure that occur during the extraction process, lignin can primarily be categorised using separation methods. In comparison to other wood components like cellulose, lignin's chemistry and structure are poorly understood. Due to its many covalent connections, resistance to degradation, heterogeneous nature, lack of selective and convenient analysis methods, and lack of efficient isolation methods, knowledge of this complex biopolymer is still delayed [8,9].

Nanoparticles have recently gained a lot of attention because of their unusual physical and chemical properties, which differ from bulk materials and single atoms. Nature's nano-dimensionality has logically sparked interest in employing nanomaterials in the realm of polymers. When good filler dispersion is accomplished and the characteristics of the nanoscale filler are significantly different or better than those of the matrix, this method is highly useful in producing high-performance composites. The dispersion of nanoparticles inside a polymer matrix has been a major problem, limiting commercial applications [10,11]. The ultrasonicated lignin nanoparticles found by Gilca [8] have a consistent spherical form. In general, lignin spherical particles are regarded to have the lowest level of toxicity because ultrasonication does not cause structural or compositional changes in nanoparticles, and its nano state is completely biodegradable [9].

Lignin's antimicrobial activity is due to its phenolic fragments, which contain a C-C double bond in the alpha and beta positions of the side chain and a methyl group in the gamma position, which damage the cell membrane and cause bacterial lysis, resulting in the release of cell contents, whereas cinnamaldehyde has the ability to penetrate bacteria's cell membrane, resulting in the reduction of cell contents. [12,13]. Antimicrobial textiles can stop off bacteria's routes to prevent bacteria from growing on textiles, effectively avoiding pathogen contact with the human body and lowering the danger of human pathogens and cross-infection rates [14]. The reactions of ortho OCH_3, $-CH_2$, and aliphatic carbonyl groups with free radicals followed a proton-coupled electron transfer mechanism, whereas the reactions of ether bonds in aliphatic side chains, carbohydrate impurities, and OCH_3 groups in C9 unit followed a sequential proton loss electron transfer (SPLET) mechanism. Both techniques were used to create the conjugated systems. Lignin's antioxidant action is primarily determined by the generation of phenoxy radicals, which scavenge free radicals Lignin antioxidant activity is influenced by extraction procedures, source species, and structure [15]. Lignin can be used as a natural antioxidant in food, medicines, cosmetics, and industrial applications. Antimicrobial fabrics can obstruct bacteria's growth paths, successfully avoiding pathogen interaction with the human body and lowering as well as minimizing the risk of human pathogens and cross-infection rates [14].

The main objective of the current study was to convert cotton plant stalks into lignin and lignin nanoparticles, then apply them to produce antimicrobial tissue (using 7 pathogenic bacteria and 5 pathogenic fungi), as well as study their antioxidant activity.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents were obtained from Sigma chemical Co. (London, Lab. Poole), England (Cairo branch). The solvents were purified and re-distilled before use.

2.2. Raw Material

Two Egyptian cotton cultivars Giza 86 and Giza 90 were planted in a field trial experiment at Giza Agricultural Research Station, Cotton Research Institute, Agricultural Research Center, Egypt. The cotton stalks were collected and preserved after cotton fiber harvisting .

2.3. Cotton stalk samples preparation

All laboratory work procedures were conducted in Cotton Seed Technology and Natural Products unit, Cotton Chemistry and Textile Fibers Department, Cotton Research Institute, Agricultural Research Center (ARC), Giza, Egypt. Cotton stalk from the two tested cultivars were dried on the land for two weeks before being chopped into splinters 2.5- 4 cm in length by then samples milled in a Hammer mill/Wood Crusher, using 0.4 mm screen, and finally, the milled samples were milled again by strong mixture mill, thereafter the samples subjected to the analysis.

2.4. Lignin extraction by organic acids (organosolv lignin)

According to Watkins [16], the formic acid/acetic acid treatment process for lignin extraction from Giza 86 cotton stalks (OL86) and Giza 90 cotton stalks (OL90) began with pulping with 85 percent organic acid (ratio of formic acid/acetic acid mixture was 70:30 by volume) and the fiber to liquid ratio of 1:8 on a hot plate for 2 h. The fibers were washed in a mixture of 80 percent formic acid and hot distilled water. In a hot water bath at 80°C for 2 h, 8 mL 35 percent H_2O_2 was added to an 85 percent formic acid/acetic acid mixture. After that, they were filtered and rinsed with hot water. The lignin was precipitated by adding distilled water (5 times the volume of concentrated liquor) and filtering the resulting solution. Finally, distilled water was used to wash the precipitated lignin before the vacuum drying over P_2O_5.

2.5. Preparation of lignin nanoparticles

Gilca [8] technique was used to produce lignin nanoparticles of various sizes. The ultrasonic horn (Sonics & Materials VC600/CV17) was used to treat 0.7 percent aqueous lignin suspensions for 60 minutes at 600 W power and 20 kHz frequency, then the lignin nanoparticles were dried in mild conditions to form Giza 86 organosolv lignin nanoparticles (OLNP86) and Giza 90 organosolv lignin nanoparticles (OLNP90).

2.6. Lignin and lignin nanoparticles characterization

2.6.1. Zeta Potential and Particle Size

Measurement of particle size of organosolv lignin (OL86 and OL90) and organosolv lignin nanoparticles (OLNP86 and OLNP90) and nanoparticles zeta potential was executed by Zetasizer nano-series (Nano ZS) instrument, Malvern, UK Company This device allows the determination of the zeta potential range (mV) from -200 to 200 mV). In brief, the size was measured three times at 25°C / 90^O scattering angle, with each measurement taking three minutes. Cumulative analysis was used to get the mean hydrodynamic diameter. The zeta potential measurements were carried out automatically with an aqueous dip cell.

2.6.2. Transmission Electron Microscopy

The morphological screening of lignin nanoparticles was determined using a transmission electron microscope (JEM-1400, JEOL model) at Cairo University Research Park. Freshly prepared Ag-NPs were placed on a glow-discharged carbon grid and air dried for minutes. The shape and surface roughness of the nanostructured samples were then investigated using a TEM.

2.6.3. Fourier Transform Infrared Spectroscopy (FTIR)

All organosolv lignin and lignin nanoparticles samples were performed in the central authority for mineral wealth labs, Giza, Egypt. The samples were prepared by milling with anhydrous potassium bromide (KBr) to form very fine powders. Then the powders were compressed into two thin pellets for analysis. The infrared spectra were recorded with a Fourier-transform infrared (FTIR) spectroscopy analyzer (Model JASCO FTIR-6100) within the scanning range 4000–400 cm^{-1}. The spectra were smoothed using 3 or 5 points and the baseline of spectra was corrected using the previously recorded spectra of the sample.

2.7. Antioxidant activity of lignin and lignin nanoparticles

DPPH radical scavenging performance of organosolv lignin and lignin nanoparticles was estimated according to **[17]** with slight modifications. (12.5µL, 25µL, 50µL, 100µL) of the sample prepared respectively. The volume was completed to 0.5 mL with methanol, and all the test tubes were vortexed well.

100µL from each bottle was taken into series of test tubes and performed the analysis in triplicates. 400µL of methanol were added into each test tube, 0.5mL of 0.1mM of methanolic solution of DPPH was also added, all the test tubes were vortexed again, then left to be stand for 30 min in the dark at RT. The transformed sample color from purple to yellow was measured in spectrophotometer at 517 nm against the blank reagent. Standard Gallic acid was used as standard antioxidant. The inhibition ratio was calculated using the following equation: DPPH scavenging performance (% inhibition) = [(-ve control absorption −sample absorption) / -ve control absorption] × 100

2.8. Organosolv lignin and lignin nanoparticles antimicrobial activity

The inhibitory effect of organosolv lignin and lignin nanoparticles was carried out for seven strains of pathogenic bacteria. Three Gram-positive bacteria; *Bacillus cereus EMCC* 1080, *Staphylococcus aureus* ATCC 13565, *Staphylococcus sciuri* 2-6 and four Gram-negative bacteria; *Salmonella typhi* ATCC 25566, *Salmonella enterica* SA19992307, *Escherichia coli* 0157 H7 ATCC 51659 and *Pseudomonas aeruginosa* NRRL B-272. The stock cultures were grown on nutrient agar slant at 37˚C for 24 h and then kept in refrigerator till use. Four fungal species were used for antifungal assay *Aspergillus flavus*, *Aspergillus ochraceus* ITAL 14, *Aspergillus niger* IMI288550, *Fusarium proleferatum* MPVP 328 as well as *Penicillium verrucosum* BFE 500. For 5 days, the stock cultures were cultured on potato dextrose agar slant at 25 °C. and then kept in refrigerator till use [18].

2.8.1 Disc diffusion technique

A loop full of each bacterial species' nutrient agar slant was inoculated in a tube containing 4 to 5 mL of tryptic soy broth (TSB). The broth culture is incubated for 2-6 h at 35˚C until the turbidity of the standard 0.5 McFarland $BaSO_4$ is fulfilled. The standard turbidity density was assessed using a 625 nm spectrophotometer. The sensitivity of lignin and lignin nanoparticles was tested with various bacterial cultures, disc diffusion method and the Kirby-Bauer technique were used **[19].** A petri dish was prepared with 20 mL of nutritional agar and bacterial cultures from TSB were added by cotton swab. sterile forceps was used to put the discs on the prepared Petri dish. DMSO used as negative control, and tetracycline (500 ppm) used as positive control then the incubation done at 37 °C for 24 h. then inhibition zone(mm) as clear area without growth was measured including the disc's diameter.

Fungal strains were grown on potato dextrose agar and incubated at 25°C for 5 days. 0.05 mL of each fungal culture was inoculated into YES medium petri dishes and spread evenly with a sterile L-glass rod. the lignin and lignin nanoparticles extracts loaded discs were placed on the seeded plates. DMSO used as negative control and Miconazole (1000 unit/mL), a commercial fungicide, was utilised as a positive control. Incubation was at 25°C/24-48 h. measurement of the inhibitory zone (mm) done for the tested fungus **[20]** were performed in 3 replicates before determining the averages.

2.8.2 Minimum inhibitory concentration determination

Tube dilution method was utilised **[21, 22]** to determine the minimum inhibitory concentration (MIC). The tested bacteria diluted by 10 mL tryptic soy broth in accordance with the 0.5 McFarland standard to achieve 10^8 CFU mL^{-1} inoculation. In culture tube with 10 concentrations of lignin extracts of 5000 ppm to 10 ppm which prepared with DMSO. The inculation was done by adding 100µl of bacteria, The suspension was incubated at 37°C for 24 h. Grouth was visible as turbidity and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract that prevented the development of the test organism, and MIC was also evaluated to the fungus using the Perrucci approach **[23].** Separately dissolved lignin and lignin nanoparticles at different concentrations Individually, they were dissolved in 0.5 mL of 0.1 percent Tween 80 (Merck, Darmstadt, Germany) with 9.5 mL of melting, 45°C, PDA and placed in a Petri dish (6 cm). A 3µL fungal suspension (10^8 CFU mL^{-1}, 0.5 McFarland standard) was centrally inoculated onto the prepared plates and For the plates were incubated at 25°C 24-48 h.

2.9. Application of lignin and lignin nanoparticles on textiles

To test the antimicrobial activity of lignin and lignin nanoparticles for economic application, the medical gauze fabric was cut with an area of 1 cm^2 and was sterilized using ultraviolet rays, so the concentration used was twice the lowest concentration (TWO FOLDED MIC) for each of the samples of Giza 86 lignin and lignin nanoparticles which reflected the

best antimicrobial inhibition zone. Lignin and lignin nanoparticles were dissolved in DEMSO, then the samples were stirred and sonicated until completely dissolved. Gauze samples were soaked in the dissolved lignin and lignin nanoparticles samples then the solvent (DEMSO) was removed by drying at 60 °C for 30 minutes (Egyptian patent No. 981/2020 from this study) . The tests were made against three pathogenic Gram-positive bacteria; *Staphylococcus aureus* ATCC 13565, *Staphylococcus sciuri* 2-6, *Bacillus cereus* EMCC 1080 and four pathogenic Gram-negative bacteria; *Salmonella enterica* SA19992307, *Salmonella typhi* ATCC 25566, *Pseudomonas aeruginosa* NRRL B-272, as well as *Escherichia coli* 0157 H7 ATCC 51659. In addition, there are 5 species of mycotoxigenic fungi were also used for antimicrobial activity test; *Aspergillus flavus* NRR 3357, *Aspergillus ochraceus* ITAL 14, *Aspergillus niger* IMI288550, *Fusarium proliferatum* MPVP 328 and *Penicillium verrucosum* BFE 500.

2.10. Statistical analysis:

Statistical significance was established using Statistic Version 9 through analysis of variance (ANOVA, one-way **analysis**) (p 0.05) and Randomized Complete Block Design (RCBD) with two factor**.** Fisher's LSD (Least significant differences) technique (= 0.05) was used to compare significant changes between treatments **[24]**.

3. Results and Discussion

3.1. Lignin and lignin nanoparticles characterization

3.1.1. Particle size distribution analysis

The data recorded in Fig. 1 (a – d) illustrates the light scattering profile for organosolv lignin (OL86, OL90) and their nanoparticles (OLNP86, OLNP90) suspensions before and after ultrasound treatment. The particle size of OL86 and OLNP86 is shown in Fig.1 (a, b), respectively. The particle size was reduced from 314.4 nm in OL86 to 197.5 in OLNP86. While Fig. 1 (c, d) shows the particle size of OL90 and OLNP90 which reduced from 318.8 to 209.9. The distribution curves could be concluded that using the ultrasonic technique was successfully reduced lignin particle size. The results in line with those found by Gupta *et al.* **[11]** and Rahman *et al.* [**25]**.

3.1.2. Zeta potential distribution

Zeta-potential is a measure of the electrostatic force on the surface of nanoparticles which indicates to some degree their stability **[26]**. The result in Fig. 2 (a, b) shows that the surfaces of OLNP86 and OLNP90 have a negative charge about -41.7 and -30.2 mV, respectively.

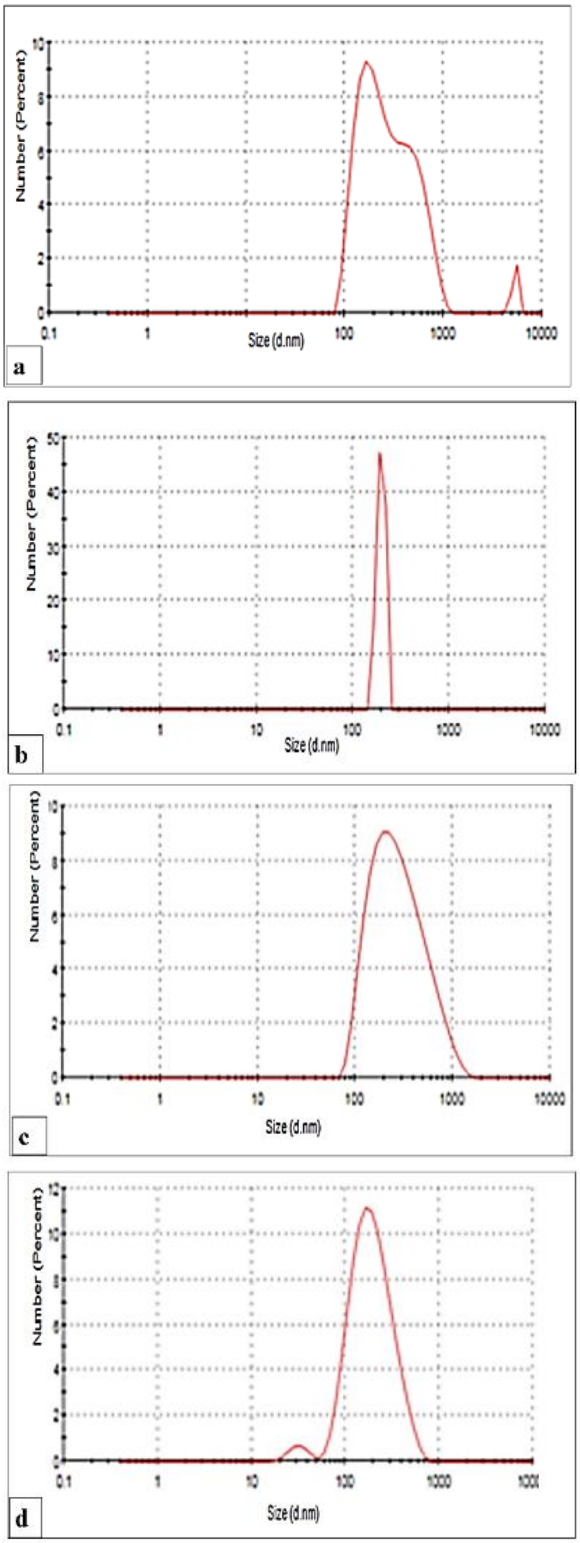


Fig. 1. Size distribution by number of OL86. (a), LNP86(b), OL90(c), and LNP90(d).

So that, Zeta potential of the nanoparticles indicates the particle electrical surface properties, the relatively high negative zeta potential hinder aggregation among the particles, which induce sufficient electrical double layer repulsion. So that, lignin nanoparticle in pure water, was quite stable, and no specific aggregation happened. These results are in accordance with Lievonen *et al.* **[27]** and Beisl *et al.* **[28].**

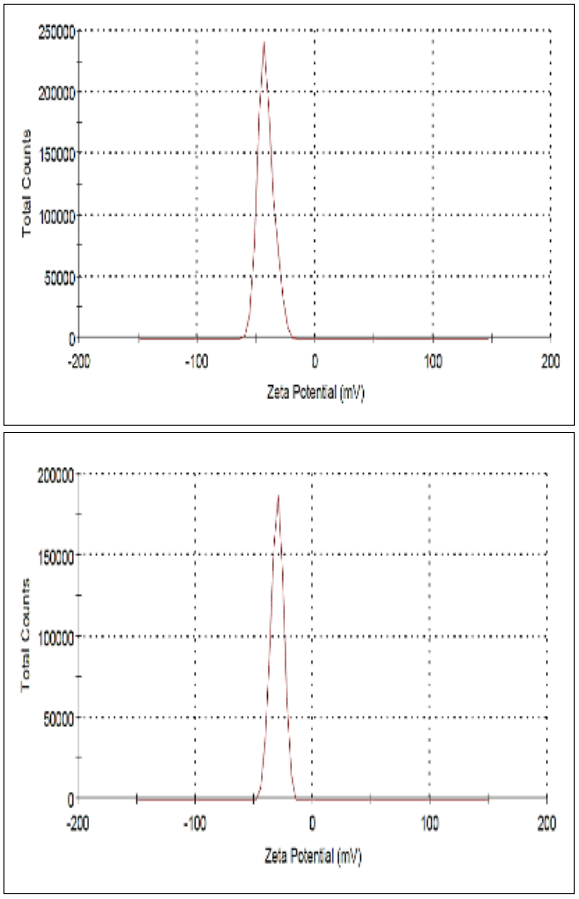
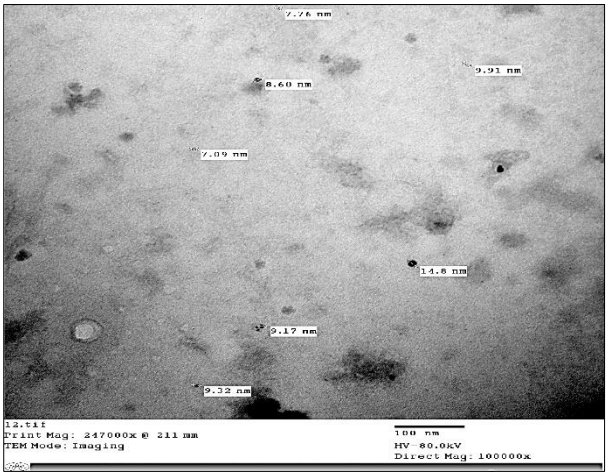


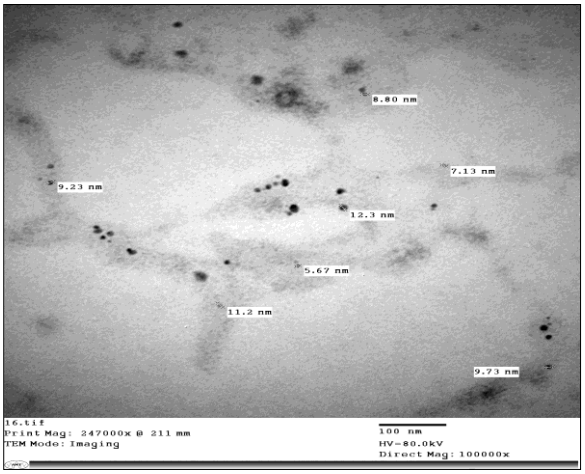
Fig. 2. Zeta potential of OLNP86 (a) OLNP90 (b)

3.1.3. Transmission electron microscopy (TEM)

The morphological characterization by Transmission electron microscopy (TEM) for OLNP86 and OLNP90 is shown in Fig. 3 (a, b) respectively. The data confirmed the presence of regular spherical lignin particles of uniform size and smooth surfaces. The obtained results showed that all the nanoparticles measured by TEM were less than 14.8 and 12.4 nm in sizes for OLNP86 and OLNP90, respectively. Their sizes for the same cultivar extracted with organic acids ranged from 5.67 to 14.8 nm. In this respect, Aforementioned results are in accordance with those found by Gilca *et al.* **[8]**, Gupta *et al.* **[11]**, Lievonen *et al.* **[27]** and Mishra and Ekielski **[29]**.



(a)



(b)

Fig. 3. Electron micrographs of OLNP86 (a) and OLNP90 (b).

3.1.4. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR Analysis is a test to study the structural analysis to specify functionality of organosolv lignin and lignin nanoparticles and study its capability for bioactive as antimicrobial and antioxidant activities. The data recorded in Fig. (4-7) illustrates the FT-IR spectra characteristic bands absorption for different presence active groups of OLNP86, OL86, OLNP90 and OL90, respectively. The result revealed that, both organosolv lignin and lignin nanoparticles had aromatic structures and the appearance of specific active groups such as C-O bands in primary alcohols, Aromatic C–O stretching vibrations, C=C of the aromatic ring, the phenolic hydroxyl groups, C-H methyl, aromatic methoxy groups and C=CH terminal. While lignin nanoparticles were distinguished by presence of effective groups which shows the presence of C=C aromatic, C≡C stretching, and O=C=O respectively,

which increases its antioxidant and antimicrobial activities. These results are in harmony with previous studies **[30,31,32,33].** The bands were ranged from 400 to 4000 cm^{-1}. It is worthwhile to mentioned that, all samples which had bands in common; 819-927, 1444-1453, 1499-1505, 1594-1600 and 1910-2093 cm^{-1} were C=C; 1213-1218 cm^{-1} were Aromatic C–O stretching; 2923-2936 cm^{-1} were CH bonds of OCHs groups; 1499-1600 cm^{-1} were C=C aromatic ring; 2913-2921 cm^{-1} were aromatic ring OCH_3; and 3227-3907 cm^{-1} were O─H alcohols and phenols. Results are close to the findings of Solaiman *et al.* **[34].** Identified types of FTIR active groups were confirmed by previous researches **[33,35,36,37,38].**

3.2. Antioxidant activity of lignin and lignin nanoparticles

The DPPH free radical assay is a spectrophotometric transfer-based electron assay used to evaluate the effect of antioxidants to reduce an oxidant that changes color when reduced **[34,39]**. The concentration of antioxidants in the sample is related to the degree of discoloration (either an increase or decrease in probe absorption at a given wavelength). The present study reports that OL86, OL90, OLNP86 and OLNP90 show free radical scavenging effect to certain extent. The study revealed that the DPPH radical scavenging activity found a dose-response relationship; the activity increased as the concentration increased for various lignin samples. The maximum antioxidant activity has been observed at (92.12±0.6%) in 100 µg/mL for OLNP90, whereas the minimum antioxidant activity was found to be has been observed at (53.75±3.4%) in 25µg/mL for OLNP86. The strongest antioxidant effectiveness for OLNP90 was found with an IC50 of 9.41µg/mL whereas the minimum antioxidant activity for OLNP86 was found with an IC50 of 11.66µg/mL (Table 1).

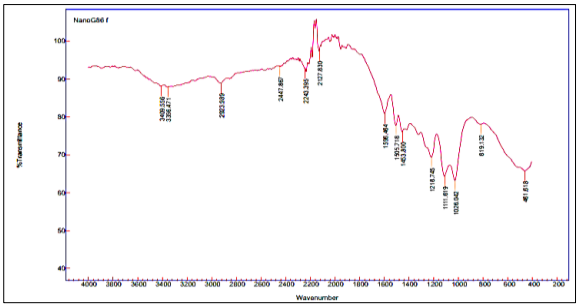


Fig. 4. FT-IR spectra of OLNP86.

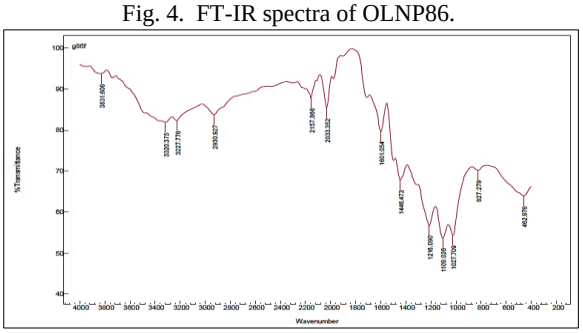


Fig. 5. FT-IR spectra of OL86.

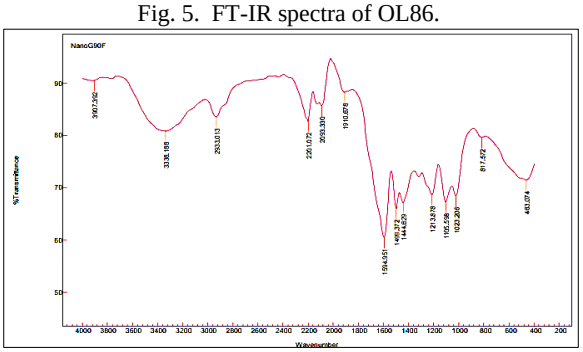


Fig. 6. FT-IR spectra of OLNP90

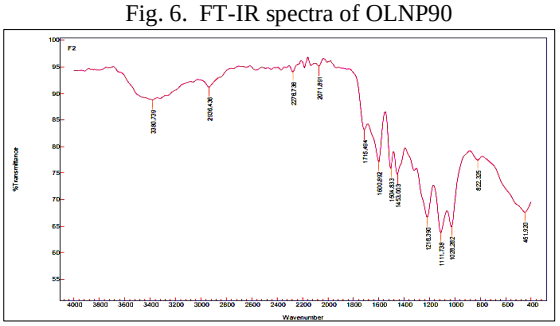


Fig. 7. FT-IR spectra of OL90.

Table 1: DPPH inhibition ratio (%) as indicators to antioxidant activity for lignin and lignin nanoparticles of Giza 86 and 90 cultivars.

	DPPH Scavenging free radicals %(Mean±S.E.)				
	Concentration(µg/mL)				
	12.5%	**25%**	**50%**	**100%**	**IC50µg/mL**
OL86	58.28^{bc}±4.6	75.86^{b}±2.04	86.44^{bc}±3.8	91.72^{b}±0.71	10.83^{ab}±1.31
OL90	65.22^{b}±1.6	70.02^{c}±0.6	82.69^{c} ±1.6	89.66^{b}±2.0	9.59^{bc}±0.24
OLNP86	53.75^{c}±3.4	60.91^{d}±3.0	68.92^{d}±2.3	75.67^{c}±5.2	11.66^{a}±3.45
OLNP90	68.25^{ab}±1.7	81.32^{a} ±3.1	88.99^{b}±1.0	92.12^{b}±0.6	9.41^{bc}±0.74
Gallic acid	77.7^{a}±0.1	83.9^{a}±0.1	99.0^{a}±0.1	99.4^{a}±0.1	8.04^{c}±0.1
LSD (0.05)	11.06	3.88	3.84	4.28	2.54

n=3, *S.E.: standard error, different subscripts differ significantly at the 5% level.

The end of oxidative propagation processes may be influenced by functional groups in lignin, such as methoxy and phenolic hydroxyl groups **[40,41]**. Lignin is a powerful antioxidant **[42].** Furthermore, lignin is made up of hydroxycinnamic acid derivatives; the antioxidant activity of hydroxycinnamic acid is determined by the number of hydroxyl groups in the aromatic ring and ortho substitutions with electron-donating methoxy groups, as shown by zeta potential tests, which revealed a value of approximately -41.7 to -30.2 mV for the studied OLNP solutions. The huge specific surface area and small spherical lignin nanoparticle size of lignin, on *the* other hand, all contribute to improved proton capacity for the phenyl group of lignin, as shown by Ponomarenko *et al.* **[15].** Extraction methods, source species, and structure all influence lignin's antioxidant activity. As a relatively safe and natural antioxidant, lignin can be added to food to prevent pathogenic germs **[14,43].** Finally, we may deduce that lignin's antioxidant activity is due to its complex structure, which comprises of aromatic rings containing 710ydroxyl and methoxy functional groups. And since organosolv lignin and lignin nano particles are compounds have weak O–H bonds and act as formal hydrogen-atom donor and the bond dissociation of the phenolic O–H group of α-tocopherol is weak and much smaller than that of the ROO–H bond of alkyl hydroperoxides. So, these antioxidants can transfer at the same moment an electron and a proton to ROO˙ and are, therefore, very versatile, because they are active also in media in which protons are not available. Actually, this kind of antioxidants is active by different degrees in all media, ranging from polar solvents to water. Lignin nanoparticles having more cleavable O–H groups than lignin on the surface, On the other hand, the numerous phenolic hydroxyl groups in lignin predominate the free-radical scavenging effect by a proton-coupled electron transfer mechanism. Moreover, ortho substitution of aromatic ring and the structure of the side-chain also significantly affect the antioxidant activity. On one hand, the ortho-substituted methoxy group with electron-donating properties increases the electron cloud density of the phenol oxygen atom, which makes the hydrogen atom detach more easily and consequently promotes the breaking of O−H bonds. As a result, lignin with higher content of phenylpropanoid units has better free-radical scavenging activity. On the other hand, the electronic effect of chemical groups in the α-position of the side chains exerts a crucial influence on the antioxidant resistance. So that behaviors can be shown in Figure (8).

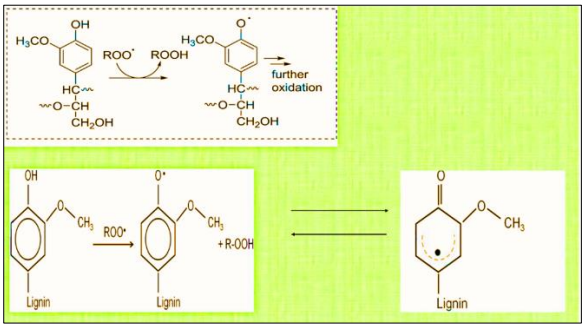


Fig. 8. Suggested mode of action for organosolv lignin and lignin nanoparticles as antioxidant agents.

3.3. Antimicrobial activity of lignin and lignin nanoparticles

Data obtained from the execution of disc diffusion technique (Tables 2 and 3) revealed that, all pathogens; 5 fungi and 7 bacteria, corresponded to lignin applicants (OL86, OLNP86, OL90 and OLNP90). The influence of lignin showed in appearance of inhibition zones for all tested pathogens. Inhibition zones for treated fungi ranged between 8.7±0.76 with *F. proliferatum* and 13.3±1.04 mm with *P. verrucosum* for OL86, and from 9.8±1.89 with *A. ochraceus* to 15.5±1.32 mm with *P. verrucosum* for OLNP86 (Table 2). As to treated bacteria, the inhibition zones ranged 9.8±0.28 with *B. cereus* and 13.0 ±0.33 mm with *S. typhi* for OL86, and from 11.0±1.00 with *S. enterica* to 14.7 ±1.28 mm with *S. typhi for* OLNP86 (Table 3). Inhibition zones for treated fungi ranged between 7.8±0.28with *A. niger* and 11.7±1.04 mm with *P. verrucosum* for OL90, and from 8.2±0.28with *A. niger* to 12.2±0.28 mm with *P. verrucosum* for OLNP90 (Table 2). As to OL90 sample, the inhibition zones ranged 9.0 ±0.48 with *Staph. Sciuri* and 11.2 ±0.76 mm with *E. coli*, and from 10.5±1.32 with *S. typhi* to 14.7 ±1.28 mm with *S. typhi for* LNP90 and from 11.0±1.00 with *S. enterica* to 11.8±0.29 mm with *P. aeruginosa for* OLNP90 (Table 3). Comparing the results showed that treatment with lignin and lignin nanoparticles had a greater effect on bacteria than on fungi.

Table 2: Antifungal activity of lignin and lignin nanoparticles against different fungal strains by Disc Diffusion Method.

Fungi	Inhibition Zone (Mean±S.E)(mm)			
	Negative control (DMSO)	Positive control (Nystatin)	OL86	OL90
A. flavus	0	16.0 ±0.76 ^{a}	8.8±0.76 ^{lm}	9.8±1.04 ^{ij}
A. ochraceus	0	10.8 ±0.36 ^{fg}	9.0 ±0.50 ^{kl}	9.2 ±1.04 $^{j-l}$
A. niger	0	8.8 ±0.14 ^{lm}	9.5 ±0.50 $^{i-k}$	7.8±0.28 ^{n}
F. proliferatum	0	11.2 ±0.58 ^{ef}	8.7±0.76 ^{lm}	8.2±0.28 ^{mn}
P. verrucosum	0	9.8 ±1.04 ^{ij}	13.3±1.04 ^{b}	11.7±1.04 ^{de}
Fungi	Negative control (DMSO)	Positive control (Nystatin)	OLNP86	OLNP90
A. flavus	0	12.8 ±0.86 ^{be}	10.7±1.04 ^{fg}	10.0±1.32 ^{hi}
A. ochraceus	0	15.4 ±1.04 ^{a}	9.8 1±.89 ^{ij}	9.2 ±0.76 $^{j-l}$
A. niger	0	15.4 ±0.76 ^{a}	12.5±1.32 ^{c}	8.2±0.28 ^{mn}
F. proliferatum	0	11.5 ±0.86 ^{e}	10.5±1.00 ^{gh}	9.0±0.50 ^{kl}
P. verrucosum	0	15.8 ±1.14 ^{a}	15.5±1.32 ^{a}	12.2±0.28 ^{cd}
LSD $_{(0.05)}$	0.65			

n= 3, *S.E.: standard error, different subscripts differ significantly at the 5% level, DMSO as a negative control, Nystatin as a positive control

Table 3: Antibacterial activity of lignin and lignin nanoparticles against different bacterial strains by Disc Diffusion Method.

Bacteria	Inhibition Zone (Mean±S.E)(mm)			
	Negative control (DMSO)	Positive control (Tetracycline)	OL86	OL90
B. cereus	0	16.6 ±0.58 ^{hi}	9.8±0.28 ^{q}	9.3 ±0.76 ^{qr}
Staph. aureus	0	18.0 ±1.04 ^{f}	10.80±1.04 ^{op}	11.0 ±1.32 $^{n-p}$
Staph. sciuri	0	17. 7±0.86 ^{fg}	11.0 ±0.50 $^{n-p}$	9.0 ±0.48 ^{r}
E. coli	0	18.8 ±1.14 ^{e}	11.2 ±0.86 $^{m-o}$	11.2 ±0.76 $^{m-o}$
S. typhi	0	15.5 ±0.50 ^{j}	13.0±0.33 ^{l}	10.7 ±0.28 $^{o-p}$
S. enterica	0	16.0 ±1.00 ^{ij}	10.7±0.76 ^{op}	10.5±1.00 ^{p}
P. aeruginosa	0	19.2 ±0.58 ^{de}	10.7±0.86 ^{op}	10.7±0.96 ^{op}
Bacteria	Negative control (DMSO)	Positive control (Tetracycline)	OLNP86	OLNP90
B. cereus	0	26.8 ^{a} ±1.04	14.2±0.84 ^{k}	11.0 ±1.00 $^{n-p}$
Staph. aureus	0	25.7 ^{b} ±0.58	13.8±0.28 ^{k}	11.5±0.50 ^{mn}
Staph. sciuri	0	26.5 ^{a} ±1.28	12.5±1.50 ^{l}	11.2 ±0.76 $^{m-o}$
E. coli	0	18.8 ^{e} ±0.86	12.8±1.89 ^{gh}	11.0±0.50 ^{np}
S. typhi	0	25.4 ^{bc} ±2.02	14.7±1.28 ^{l}	10.5±1.32 ^{p}
S. enterica	0	24.8 ^{c} ±1.15	11.0±1.00 ^{ij}	10.8±0.76 ^{op}
P. aeruginosa	0	18.9 ^{de} ±0.76	11.5±0.86 ^{d}	11.8±0.29 ^{m}
LSD $_{(0.05)}$	0.65			

n = 3 *S.E.: standard error, different subscripts differ considerably at the 5% level, DMSO as a negative control, tetracycline as a positive control.

MIC (minimum inhibitory concentration) determination exhibited in Tables 4 and 5 ranged for treated fungi from 1.5±0.36 mg/mL with *F. proliferatum to* 8.3±0.81 mg/mL with *A. niger* for OL86, and between 1.5±0.50 with *A. flavus* and 6.7±1.58 mg/mL with *A. ochraceus* for OLNP86. But as to OL90 MIC ranged from 4.2±0.36 with *A. ochraceus* to 8.3±0.58 mg/mL with *A. niger*, and OLNP90 ranged from 2.0±0.50 with *A. ochraceus* to 5.8±1.44 mg/mL with *A. niger* (Table 4). Bacteria differed in corresponding to lignin applicants as to their different sensitivity, as to treated bacteria OL86 MIC value ranged from 3.3±0.48 with *E. coli* to13.2±0.76 with P. aeruginosa, and also OL90 MIC ranged from 4.2±0.36 with *E. coli* to11.7±1.04 with *Staph. Sciuri*. The MIC of OLNP86 ranged from1.7±0.76 with *Staph. Sciuri* to

4.2±1.36 with *P. aeruginosa* and also, OLNP90 MIC ranged from 2.0±0.50 with *Staph. Aureus* to 6.7±1.14 with *Staph. Sciuri (Table 5). F. proliferatum* and *A. ochraceus* were the most sensitive fungi to lignin and lignin nanoparticles, and also *S. typhi and E. coli* were the most sensitive bacteria to lignin and lignin nanoparticles. The organic acid method cleaves carbohydrate–lignin bonds while the acid catalyst cleaves ether-linkages, resulting in purer lignin than other low-molecular-weight processes **[44]**. The results are partially compatible with de Melo *et al.* **[45]**, who found that the lignin MIC against *Aspergillus flavus* was 5 mg/mL. The ability of lignin to prevent the growth of a number of bacteria has long been known **[46]**. The phenolic components of lignin can impede the behavior of several enzymes as well as the growth of microorganisms such as *E. coli, Saccharomyces cerevisiae, Bacillus licheniformis,* and *Aspergillus niger.*

Table 4: Minimum inhibitory concentration (MIC mg/mL) of lignin and lignin nanoparticles against mycotoxigenic fungi.

Fungi	MIC mg/mL (Mean±S.E)	
	OL86	OL90
A. flavus	7.5±0.96 ^{c}	9.2±0.44 ^{a}
A. ochraceus	5.8±0.48 ^{e}	4.2±0.36 ^{g}
A. niger	8.3±0.81 ^{b}	8.3±0.58 ^{b}
F. proliferatum	1.5±0.36 ^{j}	6.7±0.48 ^{d}
P. verrucosum	8.0±0.50 ^{bc}	7.8±0.52 ^{bc}
Fungi	OLNP86	OLNP90
A. flavus	6.7±1.58 ^{d}	5.8±1.44 ^{e}
A. ochraceus	1.5±0.50 ^{j}	2.0±0.50 ^{ij}
A. niger	5.0±1.00 ^{f}	3.0±0.86 ^{h}
F. proliferatum	2.3±0.36 ^{i}	2.2±0.28 ^{i}
P. verrucosum	3.0±1.00 ^{h}	3.8±1.02 ^{g}
LSD $_{(0.05)}$	0.61	

n = 3, *S. E: standard error, alternative subscripts differ significantly at the 5% level.

Table 5: Minimum inhibitory concentration (MIC mg/mL) of lignin and lignin nanoparticles against pathogenic bacteria.

Bacteria	MIC mg/mL (Mean±S.E)	
	OL86	OL90
B. cereus	4.2±0.48 ^{h}	10.8±0.88 ^{c}
Staph. Aureus	5.8±0.48 ^{f}	5.8±0.88 ^{f}
Staph. Sciuri	6.7±0.96 ^{e}	11.7±1.04 ^{b}
E. coli	3.3±0.48 ^{i}	4.2±0.36 ^{h}
S. typhi	4.2±0.88 ^{h}	5.8±0.44 ^{f}
S. enterica	4.2±0.48 ^{h}	8.3±0.96 ^{d}
P. aeruginosa	13.2±0.76 ^{a}	10.7±1.15 ^{c}
Bacteria	OLNP86	OLNP90
B. cereus	2.3 ±0.28 $^{j-l}$	5.8±1.44 ^{f}
Staph. Aureus	3.3±0.96 ^{i}	2.0l±0.50^{m}
Staph. Sciuri	1.7±0.76 ^{m}	6.7±1.14 ^{e}
E. coli	2.8±0.89 ^{ij}	2.2 ±0.28 $^{k-m}$
S. typhi	1.8l±0.58^{m}	2.7±1.02 ^{jk}
S. enterica	4.0±1.00 ^{h}	4.2±0.76 ^{h}
P. aeruginosa	4.2±1.36 ^{h}	5.0±1.00 ^{g}
LSD $_{(0.05)}$	0.52	

n= 3, *S. E: standard error, different subscripts differ significantly at the 5% level.

The existence of a double bond at the C = C position of the side chain and a methyl group can explain the antifungal activity of lignins. Due to the existence of cinnamic acid derivatives and their known antibacterial activity, lignin's high phenolic concentration supports this hypothesis **[47,48]**. The antibacterial properties of lignin are largely defined by the side chain structure and type of functional groups of phenolic compounds. Side chains with oxygen-containing functional groups (such as hydroxyl groups, carbonyl groups, and ester groups) are less inhibitory, whereas double bonds and methyl groups increase the biocide action of phenolic compounds **[49,50,51].** The interaction of the nanocomposites with the bacterial cell wall can be governed by the lignin structure, which aids not only particle stability but also selectivity towards different types of bacteria, such as *S. aureus* (known to have a thicker peptidoglycan layer) for Gram-positive bacteria and *E. coli* (contains more fatty acids) for Gram-negative bacteria as to Rocca *et al.* **[52]**. The suggestion mode of action for the organosolv lignin and lignin nanoparticles as antimicrobial agents could be presence of the polyphenol structure of lignin in addition to the presence of the ortho-substituted methoxy functional groups with electron-donating properties may be potentially responsible for its antimicrobial activities. In addition, the polyphenolic compounds of lignin cause the cell membrane to damage and also cause the lysis of bacteria, which also causes the release of cell contents. While lignin cinnamaldehyde penetrates in the cell membrane of bacteria which causes the reduction in the intracellular pH and causes the ATP depletion, this suggestion is in accordance with Mahmood *et al.* **[13].**

3.4. Lignin and lignin nanoparticles application on textile

Separated lignin and produced lignin nanoparticles were examined as a creative material for single-use clinical textiles, especially for patients who cannot tolerate antibiotics, such as those with diabetes, high blood pressure, and renal failure. The results in Table 6 and 7 illustrate the inhibition zone for the innovative textile with understudied pathogenic fungi and bacteria, the treated textile inhibition zone for fungi ranged from 14.5 ±0.58 for OL86 with *F. proliferatum* and *F. proliferatum* to 21.7 ±1.14 mm *with A. niger* and between 10.5 ±0.36 for OLNP86 textile with *A. ochraceus* and 30.7 ±1.76mm for with *P. verrucosum* data indicated noticeable elevation for OLNP textile highest inhibition zone to 29.3%. Comparing the former data of inhibition zone for understudied bacteria, the treated textile inhibition zone ranged from 19.0±1.73 for OL86 textile with *B. cereus* and *Staph. sciuri* to 28.0±1.73 mm with *S. enterica* and between 18.5±0.96 for OLNP86 textile with *B. cereus* and 36.3±0.58 mm for *P. aeruginosa* data indicated noticeable elevation for LNP textile highest inhibition zone by 22.9%.

OL86 textile inhibition zone for gram positive bacteria ranged from 19.0±1.73 to 19.2±2.31 mm and between 19.2±2.31 to 28.0±1.73 mm for gram negative bacteria by increasing the inhibition zone for gram negative bacteria from 1.04 to 31.4%. Also, OLNP86 textile inhibition zone for gram positive bacteria ranged from 18.5±0.96 to 29.3 ±2.31 mm and between 24.7±2.88 to 36.3±0.58 mm for gram negative bacteria by increasing the inhibition zone for gram negative bacteria from 25.1 to 19.3%. OLNP86 had the wider inhibition zone on gram negative bacteria than gram positive bacteria and fungi.

Table 6: Antifungal activity of lignin and lignin nanoparticles of the innovative textile against mycotoxigenic fungi.

Fungi	Inhibition Zone (Mean±S.E)(mm)	
	OL86	OLNP86
A. flavus	14.5±0.58 ^{g}	18.5±0.92 ^{f}
A. ochraceus	21.7±1.14 ^{c}	10.5±0.36 ^{h}
A. niger	18.5±0.92 ^{e}	19.0±1.00 ^{d}
F. proliferatum	14.5±0.58 ^{g}	25.2±1.32 ^{b}
P. verrucosum	21.7±1.14 ^{c}	30.7±1.76 ^{a}
LSD $_{(0.05)}$	0.96	

n= 3, *S.E: standard error, different subscripts are significantly different at the 5% level.

Table 7: Antibacterial activity of lignin and lignin nanoparticles of the innovative textile against mycotoxigenic fungi.

Bacteria	Inhibition Zone (Mean±S.E)(mm)	
	OL86	OLNP86
B. cereus	19.0 ±1.73 ^{f}	18.5 ±0.96 f
Staph. Aureus	19.2 ±2.31 ^{f}	24.0 ±1.00 ^{e}
Staph. Sciuri	19.0 ±1.73 ^{f}	29.3 ±2.31 $^{b-d}$
E. coli	19.2 ±2.31 ^{f}	27.7 ±1.15 ^{d}
S. typhi	19.3 ±1.45 ^{f}	24.7 ±2.88 ^{e}
S. enterica	28.0 ±1.73 ^{cd}	30.3 ±0.28 ^{b}
P. aeruginosa	24.7 ±4.72 ^{e}	36.3 ±0.58 ^{a}
LSD $_{(0.05)}$	2.84	

n= 3, *S.E: standard error, different subscripts are significantly different at the 5% level.

The obtained results in this work about the organosolv lignin effect on fungi and bacteria were in conformity with those reported by Gordobil *et al.* **[53]** and Lobo *et al.* **[43].** Lignin had inhibitory effect on Gram-positive bacteria (*S. aureus*) **[54].** Also, lignin had antimicrobial against *Bacillus aryabhattai* and *Klebsiella sp.* and it can be used as potential antioxidant and antibacterial agent **[55]** and it only acts as antifungal in lower concentrations **[43].** Lignin was higher antibacterial activity than widely used antibiotics in some circumstances, that because of its strong antioxidant and polyphenolic content. Antimicrobial textiles can stop off bacteria's routes to prevent bacteria from growing on textiles, effectively avoiding pathogen contact with the human body and lowering the danger of human pathogens and cross-infection rates **[14].**

4. Conclusion

Lignin nanoparticles demonstrated a novel power to inhibit bacterial growth over time. Their biodegradability and environmental qualities open up new possibilities in the fight against bacterial infections like *Staphylococcus aureus, Staphylococcus sciuri, Bacillus cereus, Salmonella enterica,* Salmonella *typhi, Escherichia coli, and Pseudomonas aeruginosa*. Due to the hydrogen atom abstraction from the phenyl group in LNP, which would eliminate oxygen, the radical scavenging activity of the free radical DPPH overtime at varied concentrations of OLNP reveals strong antioxidation activity even at 12.5 ppm. Because of its substantial antioxidant and polyphenolic content, nanolignin had stronger antibacterial activity than commonly used antibiotics in several instances. Antimicrobial fabrics can block bacteria's pathways, preventing bacteria from growing on textiles and reducing the risk of human pathogens and cross-infection rates. The different structures and properties of lignin make the preparation of nano-lignin more challenging, posing hurdles to study into nanolignin preparation and performance. Meanwhile, it opens up more possibilities and potential for nano-multi-functional lignin and multi-field applications. A unique strategy to achieving high value-added lignin use is nanometer-sized lignin with high specific surface area and activity. Nano-lignin preparation and application are still in their infancy. Future lignin research will focus on the scalability and manufacturing of nano-lignin-based products.

5. Conflicts of interest

There are no conflicts to declare.

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