Particle Sizes-Dependent Antifungal Activity of Lignin Nanoparticles

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In this study, lignin nanoparticles (LNPs) with solvent-antisolvent ratios of 1:10, 1:20, 1:30, 1:40 and 1:50 (v/v) show mean particle sizes of 220 nm, 130 nm, 80 nm, 55 nm and 20 nm after synthesised by nanoprecipitation method in an aqueous medium. Iodo-lignin nanoparticles (ILNPs) with solvent-antisolvent ratios of 1:10, 1:20, 1:30, 1:40, and 1:50 (v/v) show mean particles sizes of 360 nm, 230 nm, 85 nm, 75 nm and 40 nm synthesised by enzymatic catalysed iodination of LNPs. ILNPs exhibited excellent antifungal efficacy against various tested fungi such as *Cunninghamella sp., Fusarium equiseti, Penicillium chermesinum, Aspergillus flavus, Aspergillus niger* and *Trichoderma piluliferum*. The maximum antifungal activity was achieved by decreasing nanosize and increasing the concentration of both LNPs and ILNPs. LNPs demonstrated inhibition effects ranging from 63.34 % to 75.71 % against *T. piluliferum, Cunninghamella sp.,* and *A. flavus*. ILNPs have a greater inhibition effect ranging from 86.33 % to 98.67 % against *A. niger, A. flavus, Cunninghamella sp., F. equiseti, P. chermesinum, and T. piluliferum*. This work has proven that LNPs and ILNPs are promising nano-fungicides for plant pathogenic fungi.

1. Introduction

Annually, plant diseases gradually decrease the productivity and quality of agricultural products. Plant diseases caused by pathogenic fungi are a global issue affecting food security (Corkley et al., 2019). *Fusarium, Aspergillus, Cunninghamella, Trichoderma* and *Penicillium* species are significant and widespread wilt pathogens affecting plant crops. These species can produce mycotoxin in several plants, vegetables and cereals (Corkley et al., 2019). There is a constant need for effective antifungal agents that are safe for the environment and human health, preferably derived from renewable resources such as lignin. Lignin is the second most abundant biopolymer after cellulose. Various functional groups of thiols, phenols and hydroxy groups make lignin an ideal precursor for functionalization with various compounds (Cui et al., 2021). According to Romainor et al., (2021), nanoparticle-based compounds offer several advantages such as increased antimicrobial and antifungal activity. There needs to be more in depth research on the antifungal study of lignin nanoparticles. According to Sharma and Sharma (2022), lignin-derived zinc oxide nanoparticles show a strong biocontrol agent against *Fusarium oxysporum* and *Fusarium proliferatum* for application in commercial plants. Interestingly, lignin functionalized silver nanoparticles show good antifungal activity against *A. niger* compared to lignin or silver nanoparticles alone (Marulasiddeshwara et al., 2017). Another study by Dos Santos et al., (2016) reported that bio-oil derived from lignin has good fungal resistance against *T. versicolor*. Using nanoparticles offers several advantages because of their high volume to surface area ratio, which allows them to effectively attack cell functions by penetrating deep inside the structure of the cell membrane (Ho et al., 2021). Nanoparticles derived from compounds such as zinc, silver and iodine have the potential as nano-fungicides in agriculture (Schneider et al., 2021). Recently, iodine and iodocompounds have shown good antifungal and antimicrobial activity at millimolar concentrations. Treatment of iodine is effective against several pathogenic fungi, including the strain of *Fusarium* species (Vasylchenko and Derevianko, 2021). Thus, our study aimed to synthesise lignin nanoparticles (LNPs) and iodo-lignin nanoparticles (ILNPs) via an aqueous-based nanoprecipitation method. ILNPs were synthesised by laccase catalysed iodination of LNPs. The antifungal

activity of both LNPs and ILNPs with different sizes and concentrations was evaluated against selected plant pathogenic fungi *Trichoderma piluliferum, Cunninghamella sp., Fusarium equiseti, Penicillium chermesinum, Aspergillus flavus* and *Aspergillus niger.*

2. Materials and method

2.1 Synthesis of LNPs and ILNPs

LNPs were prepared with different solvent-antisolvent ratio of acetone-water through a solvent-antisolvent nanoprecipitation process with slight modification (Richter et al., 2016). 2 g of alkali lignin was dissolved in acetone under ambient conditions. 1 mL of alkali lignin solution was added dropwise into ultrapure water (10 mL, 20 mL, 30 mL, 40 mL and 50 mL), which was continually stirred at 150 rpm for 1 hour using a magnetic stirrer. The resulting mixture was centrifuged, and the supernatant was discarded to obtain the regenerated LNPs. Iodination of LNPs was carried out by laccase catalysed treatment with a slight modification method (Ihssen et al., 2014). Reaction mixtures were carried in McIlvain buffer pH 5 containing 20 mM of LNPs,100 mM (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 50 mM potassium iodide (KI). LNPs suspensions were added to a final concentration of 20 mM in solvent acetone: water (1:10 v/v) and added to a mixture containing 4 U/mL of *Marasmius cladophyllus* laccase in McIlvain buffer (pH 5) with excess potassium iodide (KI). ABTS was added as a redox mediator (100 μM) to the reaction mixtures and stirred for 20 h under ambient conditions. The solid samples were collected through centrifugation and oven dried.

2.2 Characterization of LNPs and ILNPs

LNPs and ILNPs were characterised by field emission scanning electron microscope (FESEM) (JEOL JSM-6390LA) with a voltage of 10 kV. The mean particle diameter was determined by randomly measuring 50 nanoparticles using ImageJ software. Fourier transform infrared spectroscopy (FTIR) spectra of LNPs and ILNPs were obtained using a Perkin Elmer FT-IR Frontier model spectrophotometer in the region from 4,000-400 cm^{-1}.

2.3 Assessment of antifungal activity

The antifungal activity LNPs and ILNPs were evaluated against fungi such as *Cunninghamella sp., F. equiseti, P. chermesinum, A. flavus, A. niger* and *T. piluliferum*. Various concentrations and particle sizes of LNPs and ILNPs were added to the potato dextrose agar (PDA) at 6, 9 and 12 mM concentrations. The petri dish with agar was inoculated with grown fungus (4 mm diameter) at the centre of the Petri dish and incubated for 14 days under ambient conditions. The control plate was inoculated with fungi without treatment. To obtain reliable results, all the experiments were carried out in triplicate. The percentage inhibition of fungi is calculated using the proposed formula by Pandey et al. (1982).

$$\text{Percentage of inhibition} = \frac{\text{Control growth diameter - Treatment growth diameter}}{\text{Control growth diameter}} \times 100\% \qquad (1)$$

3. Result and discussions

3.1. Characterization of samples

In this study, nanoparticles are produced by precipitation through rapid solvent-antisolvent ratio of acetone-water. In this method, water act as an antisolvent without further removal of residual acetone. The main parameter affecting the size of LNPs is the solvent-antisolvent ratio of acetone-water. The change in particle diameter upon changing the solvent-antisolvent ratio is shown in Figure 1. LNPs show mean particle sizes of 220 nm, 130 nm, 80 nm, 55 nm and 20 nm with 1:10, 1:20, 1:30 and 1:40 and 1:50 solvent-antisolvent ratios. The nanoparticle sizes decrease upon increasing the solvent-antisolvent ratio. The size of nanoparticles is dependent on lignin concentration in solvent. Further increase of lignin concentration leads to particle growth. Thus, smallest size of nanoparticle (20nm) can be synthesised at lowest concentration of LNPs in solvent. Comparatively, the mean particle size of ILNPs was increased after laccase treatment. ILNPs with solvent-antisolvent ratios of 1:10, 1:20, 1:30, 1:40, and 1:50 shows 360 nm, 230 nm, 85 nm, 75 nm and 40 nm. The increase in particle growth was probably due to the functionalization of iodine to LNPs. FESEM image shows the spherical shape of lignin nanoparticles formed by the nanoprecipitation method (Figure 2). ILNPs demonstrated similar morphology (spherical shape) after iodine treatment (Figure 3). The results show that laccase iodide treatments of LNPs does not affect its nanoparticle shape. The shape of lignin nanoparticles depends on the nature of lignin and the synthesis method. The results prove that spherical lignin nanoparticle and iodolignin nanoparticle can be synthesised by the solvent-antisolvent method.
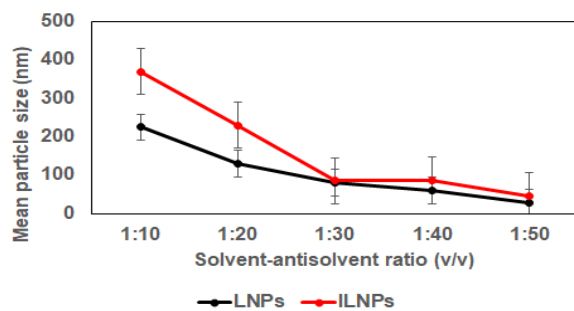
Figure 1: Mean particle size of LNPs and ILNPs at different solvent-antisolvent ratios
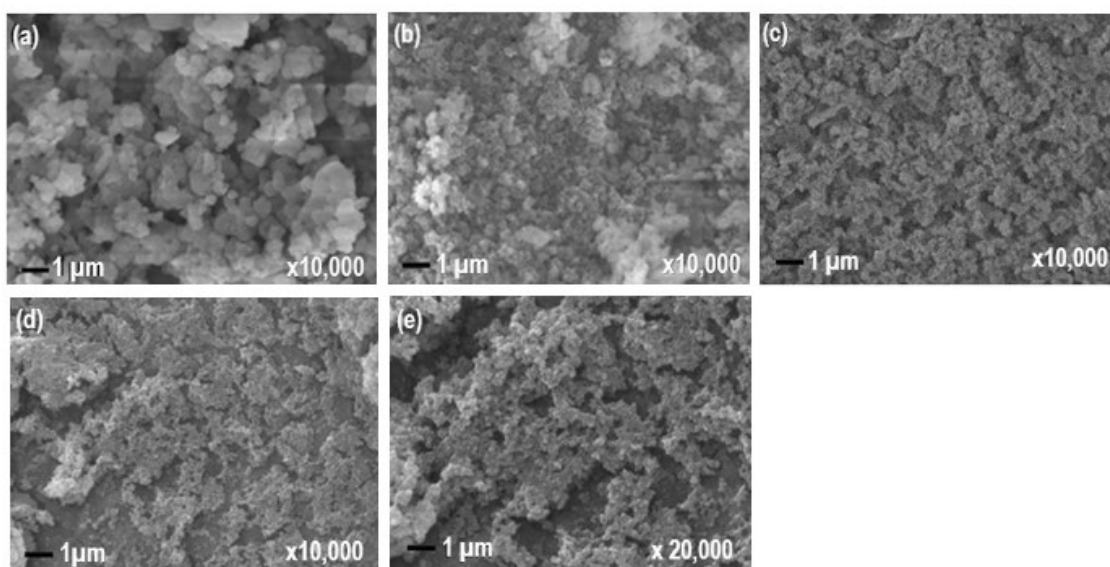

Figure 2: FESEM images of LNPs at solvent-antisolvent ratio of 1:10 (a), 1:20 (b), 1:30 (c), 1:40 (d) and 1:50 (e)
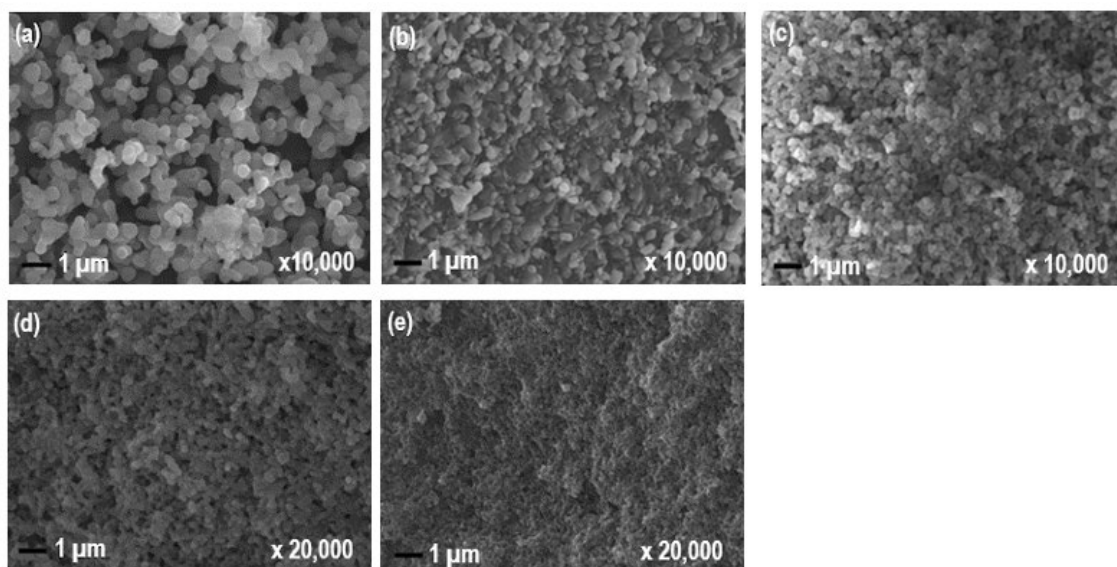

Figure 3: FESEM images of ILNPs at solvent-antisolvent ratio of 1:10 (a), 1:20 (b), 1:30 (c), 1:40 (d) and 1:50 (e)

3.2 Mycelial Growth inhibition of LNPS and ILNPs

The mycelium growth inhibition of different sizes and concentrations were analysed on PDA. When the smallest size LNPs at 20 nm was applied as treatment, the mycelium growth inhibitions were decreased at the highest concentration (12 mM). The mycelium growth of *T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger* and *A. flavus* was reduced by 48 % to 75 % at 42.52 nm with 12 mM concentration (Figure 4). Comparatively, mycelial growth inhibitions tend to decrease at highest concentration (12 mM). The mycelium growth of *T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger* and *A. flavus* was further reduced by 85% to 98% at 42.52 nm with 12 mM concentration.

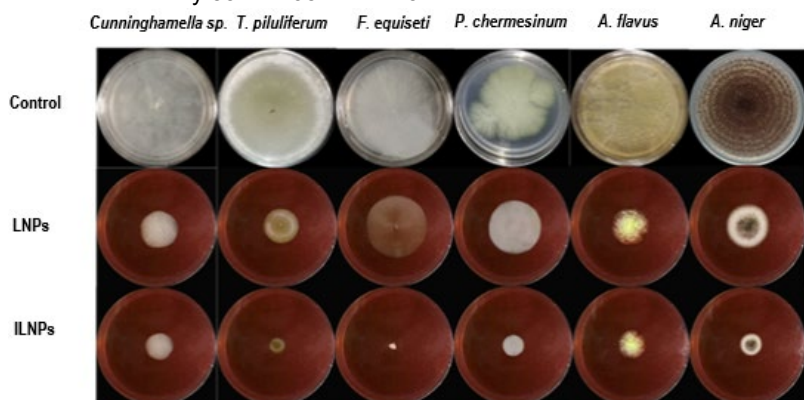*Figure 4: Mycelial growth inhibition of LNPs against T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger and A. flavus with the treatment of 12 mM*

3.3. Effect of nanoparticle size on antifungal activity of both LNPs and ILNPs

The growth of colony diameter was measured, and inhibition growth was expressed as percentage of inhibition (Table 1 and 2). LNPs demonstrated greater inhibition at the higher concentration and smaller sizes. Higher growth inhibition was observed against *T. piluliferum, Cunninghamella sp.,* and *A. flavus* at 75.71 %, 63.34% and 68.31 % with 12 mM concentration.

Table 1: Antifungal activity of LNPs at different sizes and concentrations against various pathogenic fungi

Fungi	Concentration (mM)	LNPs sizes				
		220 ± 5 nm	130 ± 5 nm	80 ± 8 nm	55 ± 5 nm	20 ± 8 nm
T. piluliferum	6	35.26± 2.1*	25.81± 1**	29.03± 1.8*	45.16± 1.5**	45.16± 1*
	9	53.61± 1**	45.48± 2**	45.16± 1.1**	60.25± 3*	61.29± 1.2**
	12	63.43± 1**	63.71± 1**	61.67 ± 1***	70.74± 2.7***	75.71± 3.3***
Cunninghamella sp.	6	56.06± 2*	41.94± 1.5**	41.94± 1*	45.16± 1.3**	40.34± 1**
	9	59.33± 1**	54.84± 1**	51.61± 1**	56.45± 2.5*	51.74± 1.6*
	12	60.33± 2*	53.23± 1.5*	54.84± 2*	58.06± 3*	63.34± 1**
A. niger	6	33.16± 1.1**	30.14± 2*	41.61± 1.2**	30.43± 1**	30.63± 1.3**
	9	40.23± 2*	38.33± 1.5*	43.13± 1**	35.62± 1.5*	39.25± 1**
	12	35.16± 1.3**	38.41± 1**	48.54± 3*	40.23± 1**	52.58± 1**
A. flavus	6	39.26± 1**	38.72± 1**	35.16± 1*	43.38± 2*	40.46± 1**
	9	51.06± 2*	48.38± 2*	46.13 ± 1.5*	60.23± 2.1***	48.72± 1*
	12	60.72± 1**	50.16±1**	51.13 ± 1*	67.23± 3.5***	68.31± 1.5***
F. equiseti	6	19.13± 1.3*	18.95± 1.5*	18.14± 2**	25.16± 1.5*	20.43± 1.5*
	9	20.13± 1**	20.25± 1**	20.25± 1**	28.13± 3*	21.52± 1**
	12	24.33± 1.5*	23.16± 1.5*	22.45± 3*	35.18± 1.5*	38.16± 1**
P. chermesinum	6	21.35± 1**	21.13± 1.8*	20.13± 1.1**	35.13± 2*	25.35± 1**
	9	31.43± 3*	29.48± 2.7*	35.13± 1**	40.58± 1**	32.16± 1**
	12	41.52± 1.3**	40.51± 3.3*	42.48± 2*	46.31± 1**	48.14± 3***

Data are given as mean± standard deviation with an average of 3 individual experiments. Significant inhibition growth compared to the control is marked by *p<0.05, **p<0.01 and ***p<0.001 (Tukey's test).

Lower growth inhibition at 6 mM against *T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger* and *A. flavus* at 25.81 %, 40.34 %, 18.14 %, 20.13 %, 30.14 % and 35.16 %. Only a few works demonstrated the antifungal activity of alkali lignin nanoparticles. According to Gordobil et al. (2018), guaiacyl and syringyl structures play a significant role in determining the antifungal efficacy of various microorganisms. Lignin was reported to have guaiacyl and syrigyl structures (Schneider et al., 2021). Therefore, the higher inhibition of lignin was due to alkali lignin's low carbohydrate and sulfur derivative content.

An increase antifungal activity was observed when lignin functionalized iodine was applied as a treatment against *T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger* and *A. flavus.* It has been shown that ILNPs show excellent antifungal activity at higher concentrations and smaller particle sizes.

Comparatively, ILNPs at 40 nm show excellent antifungal activity with 98.67 %, 89.33 %, 98.67 %, 98.57 %, 91.57 % and 87.34 % inhibition growth against *T. piluliferum, Cunninghamella sp., F. equiseti, P. chermesinum, A. niger* and *A. flavus* (Table 2).

These results agree with other authors. It was reported that iodine compounds have good antifungal activity at millimolar concentration against plant pathogenic fungi *Acremonium sp.* and *Fusarium sp.*, (Vasylchenko and Derevianko, 2021). Another study also reported that iodine is effective against *Candida sp.* (Chesnay et al., 2021). The main reason is the release of membrane-associated toxicity effect and the release of iodine from iodocompound. The biocidal effects of iodine against fungi will first rupture the cell wall, leading to the leakage and shrinking of the cytoplasmic structure. Finally, the coagulation of proteins and precipitation of mineral crystals occurs due to dehydration of the cytoplasm (McDonnell et al., 1999). Similarly, treatment of ILNPs increased the lipophilicity of fungi cell membranes due to the release of free iodine from ILNPs.

Table 2: Antifungal activity of LNPs at different sizes and concentrations against various pathogenic fungi

Fungi	Concentration (mM)	LNPs sizes 360 ± 9 nm	230 ± 8 nm	85 ± 9 nm	75 ± 5 nm	40 ± 5 nm
T. piluliferum	6	48.76 ±1.3*	53.34 ±1.3**	55.71 ±1.3*	62.43 ±1.2*	55.16 ±1**
	9	78.18 ±5*	82.34 ±3.3*	86.18 ±2**	93.15 ±1***	92.51±1.3***
	12	94.57 ±2***	95.47±1.2***	96.43 ±1.2**	98.57 ±1***	98.67±1.3***
Cunninghamella sp.	6	64.33 ±1.3**	66.34 ±2*	67.57 ±2**	69.74 ±1**	69.13 ±2***
	9	78.33 ±2**	81.43 ±1**	83.15 ±1**	83.34 ±1.3**	83.13 ±1**
	12	81.15 ±3**	85.25 ±3.3**	86.15 ±5***	89.25 ±1***	89.33±1.3***
A. niger	6	48.25 ±1**	50.13 ±3*	61.23 ±1**	64.13 ±1.5*	63.43 ±1.3**
	9	63.43 ±3.3*	66.23 ±1**	67.13 ±5*	71.52 ±5*	70.34 ±3*
	12	80.43 ±2*	84.34 ±2**	86.74 ±1***	91.57 ±1***	89.57 ±1***
A. flavus	6	48.38 ±1**	53.86 ±2*	52.15 ±3.3**	55.33 ±1**	55.86 ±2*
	9	63.43 ±3.3*	65.43 ±1**	65.43 ±1**	68.43 ±1.2*	65.43 ±1.3*
	12	81.76 ±2*	83.57 ±5*	85.57±2.3***	87.34±2.3***	86.33 ±1***
F. equiseti	6	70.43 ±3*	73.74 ±2*	73.34 ±1.3**	83.33 ±1**	81.43 ±1**
	9	91.43 ±2.3**	93.47 ±2.3**	93.33 ±2***	95.18±2.3***	95.18 ±3***
	12	96.33 ±1**	98.33 ±1**	98.18 ±1**	98.67 ±1**	98.67 ±1.3*
P. chermesinum	6	68.33 ±1.3*	71.18 ±3*	73.33 ±2*	74.86 ±1**	72.14 ±1**
	9	81.13 ±1**	84.18 ±1**	91.31 ±1**	91.75 ±3.3**	93.23 ±2***
	12	93.53±1.5***	94.43 ±1***	96.89 ±1***	95.18 ±2**	98.57 ±1***

Data are given as mean± standard deviation with an average of 3 individual experiments. Significant inhibition growth compared to the control is marked by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Tukey's test).

4. Conclusions

In summary, lignin nanoparticles and iodolignin nanoparticles were successfully prepared by a simple nanoprecipitation method. The spherical shape of lignin nanoparticles was prepared through a solvent-antisolvent process followed by oven drying. Iodine was successfully incorporated onto the surface of lignin nanoparticles by laccase catalysed reaction. The morphology of iodolignin nanoparticles is similar to the lignin nanoparticle under FESEM analysis. In relation to the mycelial growth inhibition test, efficient inhibition of LNPs and ILNPs are verified. Lignin nanoparticles show good antifungal activity against *A. flavus and T. piluliferum.* In combination with iodine, iodolignin nanoparticles showed greater antifungal activity against *T. piluliferum, Cunninghamella sp. A. niger, A. flavus, F. equiseti* and *P. chermesinum.* Since iodolignin nanoparticles show excellent antifungal activity, further research is required to determine their toxicity before its application as a potential nano-fungicide in agriculture.

Acknowledgment

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