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Biosynthesis of ZNO nanoparticles (ZNO-NPS) from potential fungi and their antifungal activity against *Helminthosporium oryzae*

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Abstract
In the present investigation stated that the nano science is a new interdisciplinary subject that depends on the fundamental properties of nano size objectives. The present study the biosynthesis of nano particles from some specific fungi like *Aspergillus Niger* and *A. flavus*, were performed. It was12.12±3.12 and 15.2±4.65 µg/ml found to be recognized with respective fungi. The effect of synergetic effect of antagonistic properties of potential fungi *A. flavus* and ZnO-NPs was approached and maximum antagonistic effect in 75µl:30µl was 14.72±6.02mm zone of inhibition recorded and minimum antagonistic effect of 25µl:10µl concentration of *A. flavus* secondary metabolites with ZnO-NPs was 08.07±0.09mm zone of the inhibition represented against *Helminthosporium oryzae* pathogen. The *A.niger* potential fungi with silver nano particles of synergistic effect were maximum at 75µl:30µl concentration in 12.10±4.18mm zone of the inhibition whereas minimum 25µl:10µl was 09.05±1.01mm zone of inhibition determined against *Helminthosporium oryzae* respectively. In the case of synergistic effect of potential fungi *A. flavus* with *A. niger* +ZnO-NPs at 75µl:30µl was 15.45±5.91mm zone of inhibition recognized and more effective antagonistic activity respectively whereas minimum antifungal activity of 50µl:20µl concentration was 10.03±2.08 from this work, the synergistic effect of *A. flavus, A. niger* and ZnO-NPs was excellent antagonistic properties against *H. oryzae*. Hence, the *A. flavus and A. Niger* with silver nano particles were excellent candidate for biocontrol agent against *H. oryzae.*

Keywords: *A.niger*, *A.flavus*, zinc oxide nanoparticles, antimicrobial activity, synergistic effect

Introduction
The advancements in science and technology, research scientist's attempts for creating nanoparticles of size within 100 nm (Wu, *et al.*, 2011) [9] due to its wide range of benefits (Mahalambi, *et al.*, 2012). It can be synthesized through the biological, chemical and physical methods. Among the methods, biological methods seems effective and ecofriendly one because of other two methods has some poisonous compounds confines their application. The, researchers tend to exploit biological methods by developing high-yielding, low-cost and non-toxic nanoparticles.
The biological method was applied with the help of biologically active (Zhao, *et al.*, 1997) [20] products of bacteria, fungi, plants, and yeasts represented the excellent sources for the production of nanoparticles (Hagfeldt, *et al.*, 2000) [8], Mostly fungi were chosen instead of bacteria because of their tolerance, better metal bioaccumulation ability and high binding capacity. There are wide applications of fungi as they produce huge enzymes, ease in the scale-up process, economic viability, and ease in handling the biomass. The ZnO nanoparticles were synthesized by a chemical route and displayed certain characteristics defined by synthesis conditions. ZnO-NPs were used to carry out a systematical analysis of different concentrations and determine the antifungal effect on *A.flavus* and *A.niger*. The study also evaluated the effect of varying synthesis of parameters including initial concentration of precursor on the fungicidal capacity of nanoparticulate ZnO. The nanotechnology has become increasingly important in the biomedical and pharmaceutical areas as alternative antimicrobial strategy due to the re-emergence of infectious diseases and the appearance of antibiotic-resistant strains especially within. Nanoparticles (NPs) are typically no greater than 100 nm in size and their biocidal effectiveness is suggested to be owing to a combination of their small size and high surface-to-volume ratio which enable intimate interactions with microbial membranes (Allaker, 2010; Morones *et al.*, 2005) [3]. In addition, inorganic antifungal agents such as metal and metal oxides are advantageous compared to organic compound due to their stability (Sawai, 2003; Sondi and Sondi, 2004) [17]. Among these metal oxides, ZnO has attracted a special attention as antifungal agent. For instance, ZnO inhibits the adhesion and internalization of enterotoxigenic *A. Niger* and *A.flavus*, into enterocytes (Roselli *et al.*, 2003) [16]. In addition, ZnO nanoparticles (ZnO-NPs) exhibited antifungal activity and can reduce the attachment and viability of microbes on biomedical surfaces (Brayner *et al.*, 2006 and Yamamoto, 2001) [4, 19].
ZnO have attracted interest as antimicrobial agents because of their safety and stability (Gómez-Ortíz, *et al.*, 2014) [7]. However, several physical and chemical methods are used for the synthesis of NPs and may provide nanoparticles of different morphologies and sizes. The synthesized ZnO powder using both co precipitation and hydrothermal methods, while the MgO powder was only prepared by co precipitation. The ZnO and MgO nanoparticles with different sizes and morphologies and determined their antifungal properties against the causal agent. The study, the

investigated the antifungal activities of ZnO nanoparticle against two important opportunistic fungi, *A.flavus* and *A.niger* which known to be resistant to many of antifungal agents (LilliHe, *et al.*, 2011) [12].

The development of vaccines and new antimicrobial agents has not kept pace with resistance; therefore, the search for other methods of therapy such as synergistic combinations. Combination therapy is applied with the intention of expanding the antimicrobial spectrum, minimizing toxicity, preventing the emergence of resistant mutants during therapy and obtaining synergistic antimicrobial activity (Eliopoulos and Moellering, 1991) [5]. The increased clinical response with combination therapy is explained due to synergism between the antibiotics used. Synergism of a combination of antibiotics can be stated as fractional inhibitory concentration indices (FICI) derived from a checkerboard titration. Synergism has been defined as a phenomenon in which two different compounds are combined to enhance their individual activity. If the combination results in worsening effect, it is called antagonism. An effect which is less than synergistic but not antagonistic activity termed as additive or indifference (Rani *et al*. 2009) [15].

Materials and Methods

Synthesis of ZnO nanoparticles from medicinal plants (Zare *et al.*, 2017)

The ZnO NPs were prepared successfully using *A.flavus* and *A.niger* separately extracted by the method of Elham Zare *et al*, 2017.The ZnO NPs were synthesized using 0.01M, 0.05M, and 0.1M $Zn(NO_3)_2 \cdot 6H_2O$ solution in 90 mL distilled water; then, 10 ml of the prepared *A.flavus* and *A.niger* extract was added drop wise into the zinc nitrate solutions under constant stirring at 60°C for 5 hours to achieve a complex formation, and NaOH (5M) was added to the solution during stirring process to adjust the pH. Both the extract (control) and the mixture (zinc nitrate+extract) were then claimed at 350°C±10°C for 2 hours in a muffle furnace to obtain ZnO-NPs.

Determination of antifungal activity of Zinc oxide nano particles against fungi (Perez *et al.*, 1990)

Preparation of culture inoculum

The stock cultures of fungi (*A.niger* and *A.flavus*,) used in this study was maintained in potato dextrose agar at 4°C. Inoculum was prepared by suspending a loop full of fungal cultures into 10 ml of potato dextrose agar and was incubated at 35°C±2°C for 2 days.

Agar well-diffusion method

Agar well-diffusion method was followed for antifungal activity. Potato dextrose agar (PDA) plates were swabbed (sterile cotton swabs) with 24 hours culture and 48 hours old - broth culture of respective fungi was inoculated. Agar wells (5mm diameter) were made in each of these plates using sterile Cork borer. About 100µl of solvents ZnO-NPs extracts were added using sterilized dropping pipettes into the wells and plates were left for 1hour to allowed a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions The petri plates were incubated in an upright position at 37±2°C for 48 h for fungal pathogens.

After the incubation period the presence or absence of inhibition zone were determined. The inhibitory zone around the well indicated the absence of tested organism and it was reported as positive and absence of zone as negative. The diameters of the zones were measured using diameter measurement scale. The were maintained and the average values were recorded for antifungal activity.

Synergistic Inhibition Effect of ZnO-NPs and potential fungi (Weidong Huang, *et al*., 2020) [18]

The 50% inhibition concentration (IC_{50}) of ZnO-NPs and potential fungi was measured through a mycelium growth rate method. A different mixed proportion was set as 25:10, 50:20 75:30 and 100:40. All mixtures were prepared fresh and blended thoroughly for 5 min. The synergistic activity assessment (toxicity ratio) of ZnO-NPs and potential fungi was determined by the following equations

1. Actual inhibition rate = [(diameter of control colony-diameter of treatment colony)/ (diameter of control colony- diameter of fungus block)] 100%
2. Theoretical inhibition rate = (actual inhibition rate of A at medium concentration. Percentage of A + actual inhibition rate of B at medium concentration percentage of B) 100%
3. Toxicity ratio= actual inhibition rate/ theoretical inhibition rate

The combination activity showed synergistic effect when the toxicity ratio was greater than 1; it showed antagonistic effect when the toxicity ratio was less than 1; it showed additive effect when the toxicity ratio was almost equal to 1.

Result and Discussion

The antagonistic fungi *A.niger* recorded in 100µl of zone of inhibition was 12.12±3.12 mm the zone of inhibition 15.2±4.65mm was *A.flavus* and the zone of inhibition *were* recorded in respectively (Table 1).

Synthesis and application of nanomaterials is in the limelight in modern nanotechnology. The silver nanoparticles by the reduction of the aqueous zinc metal ions during exposure to the potential fungi. Formation of zinc oxide nanoparticles were monitored by UV-vis spectroscopy. Reduction of the $AgNO_3$ ions and formation of zinc oxide nanoparticles were completed in 60 min of reaction. The colourless solution changed into brownish yellow colour which indicates the formation of zinc oxide nanoparticles. The UV-vis spectra showed no evidence of absorption in the range of 400-800 nm for the potential fungi and the potential fungi solution exposed to $AgNO_3$ ions showed a distinct absorption at around 434 nm which corresponds to SPR of zinc oxide nanoparticles established at 420 nm (Mulvaney, 1996) [13].

Table 1: Biosynthesis of zinc oxide nanoparticles

S.No	Name of the fungi	Quantity (µg/ml)
1	*Aspergillus niger*	12.12±3.12
2	*A.flavus*	15.2±4.65

The compound of different antimicrobial substances in consideration of environmental pollution and drug resistance. The biosynthesized Ag-NPs and epoxiconazole could be combined amicably, and there exhibited no obvious antagonistic effect among these mixed proportions. The prominent synergistic antifungal effect occurred at different concentration of secondary metabolites of *A.flavus*

+ZnO-NPs was 25:10 in 09.04±1.05, 08.07±0.09, 09.56±1.69, 10.32±1.21 and 10.01±1.06mm minimum zone of the inhibition recorded respectively. Different concentration of secondary metabolites *A. flavus* +ZnO-NPs was 50:20 in 11.09±1.08, 11.10±1.09, 12.15±2.96, 12.74±2.26 and 11.32±2.69mm zone of the inhibition against *Helminthosporium oryzae* recorded respectively. The different concentration of secondary metabolites of *A. flavus* +ZnO-NPs was 75:30 in 13.45±5.91, 13.86±5.59, 14.72±6.02, 14.56±6.32 and 15.02±6.98 mm maximum zone of the inhibition against *Helminthosporium oryzae* found to be recorded in respectively. Different concentration of secondary metabolites of *A.flavus* +ZnO-NPs was 100:40 in 12.36±3.36, 12.45±2.65, 11.09±1.96, 10.25±2.23 and 10.96±2.56mm zone of the inhibition against *Helminthosporium oryzae* recorded respectively(Table 2)

Table 2: Synergistic effect of *Aspergillus flavus* with ZnO-NPs against *Helminthosporium oryzae*

Different conc. of sec. metabolites of *A.flavus* +ZnO-NPs(µl:µl)			
25:10	**50:20**	**75:30**	**100:40**
Zone of inhibition (mm)			
09.04±1.05	11.09±1.08	13.45±5.91	12.36±3.36
08.07±0.09	11.10±1.09	13.86±5.59	12.45±2.65
09.56±1.69	12.15±2.96	14.72±6.02	11.09±1.96
10.32±1.21	12.74±2.26	14.56±6.32	10.25±2.23
10.01±1.06	11.32±2.69	15.02±6.98	10.96±2.56

Standard deviation ± Standard error

Different combinations of ZnO nanoparticles with different concentration of secondary metabolites *A. Niger* +ZnO-NPs 25:10 was 10.04±2.05, 09.05±1.01, 09.86±1.69, 10.02±2.01 and 11.06±5.06 mm minimum zone of the inhibition recorded in respectively. Different concentration of secondary metabolites *A. Niger* +ZnO-NPs was 50:20 in 09.19±2.05, 10.56±2.16, 11.17±2.35, 11.12±2.29 and 11.53±2.54 mm zone of the inhibition against *Helminthosporium oryzae* suppressed respectively. Different concentration of secondary metabolites *A. Niger* +ZnO-NPs of 75:30 µl12.16±3.11, 11.45±2.19, 10.89±2.12, 11.78±3.22 and 12.10±4.18mm maximum zone of the inhibition against *Helminthosporium oryzae* recorded respectively. The *A. Niger* secondary metabolites +ZnO-NPs was 100:40 in 10.46±2.36, 09.69±2.65, 09.19±2.56, 09.15±3.23 and 10.86±3.36mm zone of inhibition against *Helminthosporium oryzae* recorded respectively (Table 3)

Table 3: Synergistic effect of *A. niger*sec. Metabolites with ZnO-NPs against *Helminthosporium oryzae*

Different conc. of sec. metabolites of *A.niger* +ZnO-NPs(µl: µl)			
25:10	**50:20**	**75:30**	**100:40**
Zone of inhibition (mm)			
10.04±2.05	09.19±2.05	12.16±3.11	10.46±2.36
09.05±1.01	10.56±2.16	11.45±2.19	09.69±2.65
09.86±1.69	11.17±2.35	10.89±2.12	09.19±2.56
10.02±2.01	11.12±2.29	11.78±3.22	09.15±3.23
11.06±5.06	11.53±2.54	12.10±4.18	10.86±3.36

Standard deviation ± Standard error

The biosynthesized ZnO nanoparticles of different concentration of secondary metabolites *A. flavus* + *A. Niger* +ZnO-NPs of 25:10µl was 11.04±3.05, 11.05±3.01, 11.86 ±2.69, 11.02 ±2.21 and 11.01±3.06mm minimum zone of inhibition against *Helminthosporium oryzae* suppressed respectively. The fungal secondary metabolites *A. flavus* + *A. Niger* +ZnO-NPs of 50:20µl was 10.03±2.08, 11.10±3.09, 12.15±3.96, 12.74±3.26 and 12.32±3.69 mm zone of the inhibition against *Helminthosporium oryzae* were suppressed drastically. The fungal secondary metabolites *A. flavus* + *A. Niger* + ZnO-NPsof 75:30µlwas 15.45±5.91, 14.86±5.59, 12.72±3.02, 13.56±4.32 and 13.02±4.98 mm maximum zone of the inhibition against *Helminthosporium oryzae*were performed. The fungal secondary metabolites *A. flavus* + *A. Niger* +ZnO-NPs of 100:40 concentration was 11.36±3.36, 12.45±3.65, 11.09±3.56, 12.25±3.23 and 11.96±3.36 mm zone of the inhibition against *Helminthosporium oryzae* were respectively good results observed respectively (Table 4).

The antimicrobial activity showed that synergistic for conjugation of ZnO-NPs and antifungal agents reported previously (Fayaz, *et al* 2010 and Hwang, *et al.*, 2012). However, it diversified when ZnO-NPs combined with fungi stats, additive and antagonistic effects were also found when ZnO-NPs were mixed with propineb and potential fungi against *Helminthosporium oryzae*,

Table 4: Synergistic effect of potential soil fungi with ZnO-NPs against *Helminthosporium oryzae*

Different conc. of sec. metabolites of *A.flavus* with *A.niger* +ZnONPs(µl: µl)			
25:10	**50:20**	**75:30**	**100:40**
Zone of inhibition (mm)			
11.04±3.05	10.03±2.08	15.45±5.91	11.36±3.36
11.05±3.01	11.10±3.09	14.86±5.59	12.45±3.65
11.86±2.69	12.15±3.96	12.72±3.02	11.09±3.56
11.02±2.21	12.74±3.26	13.56±4.32	12.25±3.23
11.01±3.06	12.32±3.69	13.02±4.98	11.96±3.36

It can be concluded that the effect of specific fungal secondary metabolites and zinc nano particles have enormous potential for antimicrobial properties against clinical microbes. This process of crude drug and ZnO-NPs production is ecofriendly as it is free from solvents and characterized in specific compounds in future.

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