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Preparation of a Nanohybrid Antifungal from Griseovulvin and Zinc Oxide and Determination of Inhibitory Activity against Dermatophyte Isolates

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Abstract
In the current study Gri-ZnO,a new nanohybrid antifungal, was prepared using Sol-Gel ion exchange between the antifungal griseovulvin (Gri) and zinc oxide. Gri-ZnO was confirmed by changes in the FT-IR and x-ray spectrums, compared with zinc oxide. New nanodimensions were also observed in atomic force microscopy. The antifungal activity of free griseovulvin and Gri-ZnO was studied against 12 patient dermatophytes belonging to three species, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum canis*. While some of the isolates showed resistance to both Gri and Gri-ZnO others were susceptible, with average inhibition zone sup to15 mm.
Key words: Nanohybrid, Zink Oxide, Griseovulvin, Biological Activity

1. Introduction

While people are exposed to fungal microorganisms regularly, only a small proportion of species may be considered pathogenic. Most fungal infections afflict the epidermis, comprising the coetaneous and subcutaneous tissues. The coetaneous fungi are keratinophilic and so generally infect skin keratins, afflicting millions of people and animals globally [1]. A prominent sourceof keratinophilic pathogensis the genus *Trichophyton*, which is one of the most widely distributed of the human and animal dermatophytes[2,3], most commonly leading to Tinea. The genera most commonly associated with Tinea include *Trichophyton*, *Epidermophyton* and *Microsporum* [4]. Many studies show that such dermatophytes are transmitted directly and indirectly through contact with dogs, as they are part of the normal flora in canines but lead to infection in humans [3].

Recently the prevalence of dermatophyte infection increased dramatically to 20 -25%of the world population, which coincided with fungal resistance to known antifungals. This has led to more studies being conducted to find new antifungalsthat can replace modern treatments that fail in the plight of growing resistance [5,6].

The antifungal griseovulvin belongs to the hetrocyclicbenzofuran group and was discovered in 1939 after it was isolated from *Penicillium griseofulvum*. Griseovulvin came to be considered an important antifungal, particularly since it has low toxicity and can therefore be taken orally or asan active in skin ointments [7,8].

Griseovulvinis a crystalline white-yellow powder with formula $C_{17}H_{17}ClO_6$ and 352.767g/mol molecular weight. It is soluble in ethanol, methanol, dimethyl- and form-amide, while partially soluble in water [9,10].

Recently the nanotechniquehas achieved popularity in medicine and pharmacy research and is now considered to have enormous potential for diagnosis and treatment of diseases [11]. In the current study the nanotechnique was used to prepare a nanoantifungal with the objective of enhancing antifungal activity. More specifically, the current study aimed to prepare a nanohybrid antifungal from griseovulvin (also spelled griseofulvin) and restore its efficacy in the face of growing resistance.

2. Materials and Methods

2.1 Preparation of antifungal nanoparticales from zinc oxide and griseovulvin by Sol gel ion exchange

The antifungal ZnO nanoparticles were prepared from griseovulvin and zinc oxide layers by the sol-gel ion exchange method which was modified from Kolekar et al., [12]. Briefly, 50 ml of griseovilvun (10 mg/ml) was added drop by drop to a zinc oxide solution (1 g of zinc oxide in 50% ethanol (20 mg/ml). Then, the solution was stirred at room temperature for 2hr. After that, the mixture was incubated with shaking at 37C^{o}for 18 hr then put in an incubator at 40 C^o for 24 hr. The solution was then centrifuged at 5000 rpm for 20 minutes and the precipitant was washed with deionized water several times then dried at 40C^o. Finally, the dried product (precipitate) was grinded and stored in refrigerator.

2.2 Nanohybrid antifungal diagnosis (screening).

Several methods were used to confirm the success of the nanohybrid antifungal (Gri-ZnO) formation, which included spectral methods and atomic force microscope (AFM).

2.2.1 Nanohybrid antifungal diagnosis using infrared spectrum (FT-IR)

A tablet was prepared from the nanohybrid and potassium bromide (KBr) then finely ground (crushed). The infrared spectrum was measured at wavelengths ranging from 400-4000 cm^{-1}.

2.2.2 Nanohybrid antifungal diagnosis using x-ray diffraction (XRD)

The nanohybrid antifungal Gri-ZnO was characterized by XRD to clarify the layer thickness' differences (d) before and after antifungal insertion using Bruker $n\lambda = 2d\text{Sine}$ equation to calculate layer's thickness (d).

2.2.3 Nanoantifungal using atomic force microscope (AFM)

The AFM was used to test the nanohybrid antifungal Gri-ZnO and measured the diameter, size, and nanoparticles' grouping. The crystallinity index was calculated using the equation: Crystallinity Index = D_p / L [13].

2.2.4 Antifungal activity of free Griseovulvin and nonohybridGri-ZnO using well diffusion assay against dermatophytes .

The antifungal inhibition activity of free griseovulvin and Gri-ZnO against 12 strains of dermatophytes were measured in a well diffusion assay as described byEgorove [14]. Also the minimum inhibition average of the free griseovulvin and nanohybrid against the isolated dermatophytes was determined using several concentrations (5, 1, 0.5, 0.25, 0.1 and 0.01 mg/ml).

3- Results and Discussion

3.1Nanohybrid antifungal screening

3.1.1Nanoantifungal of Griseovulvinscreening usingInfrared spectrum

The free Griseovulvin showed many significant peeks (bands) with specific frequencies as shown in Figure 1. The appearance of bands at the frequency 3392.90 cm^{-1} referred to the stretch vibration of the aromatic C-H bonds. In addition, the C-H

stretched at frequencies 2847.03 and 2941.54 cm^{-1} is from the alkene bondon at the aliphatic side of the molecule. While the bands 16660.77 and 1708.99 $cm^{-1}$are indicative of the vibration of the carbonyl groups (C=O). The appearance of the band at 1460.16 cm-1 referred to the vibration at the bending C-H backbone.

3.1.2 Nanoantifungal of zinc oxide layers screening using infrared spectrum

The zinc oxide layers showed a band at400-500cm^{-1} as a result of vibration at the metal ZnO bond (Figure 2).

3.1.3 **Nanohybrid antifungal (Gri-ZnO) screening using infrared spectrum**

Figure 3 shows that the Gri-ZnO complex has formed which is indicated by a substantial reduction of the bands at the frequencies 3396.76$cm^{-1}$and 2943.47 cm^{-1}.This indicates proton substitution in the formation of the Gri-ZnO complex. The two bands at 1618.33 and 1707.06 $cm^{-1}$are retained since the carbonyl groups are unchanged in the reaction. The bands at 428.21 and 439.78 $cm^{-1}$are also retained since the stretch on the metal bond in Zn-O is still there[15].

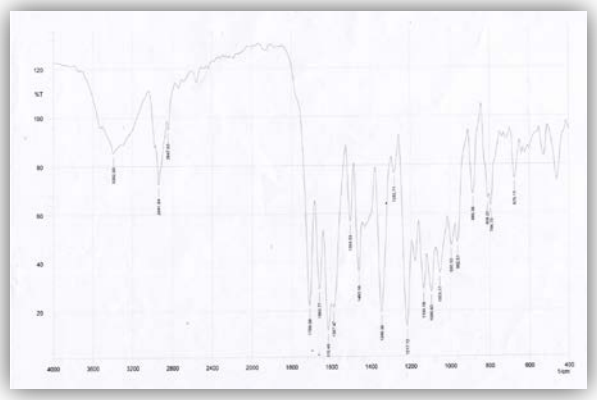


Figure 1 - Infrared spectrum of Griseovulvin

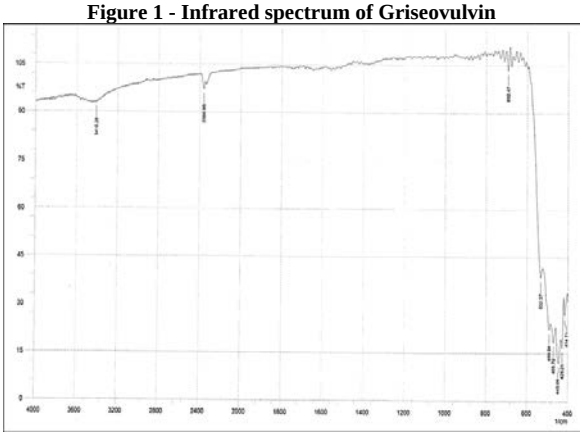


Figure 2 - Infrared spectrum of Zinc-Oxide layers.

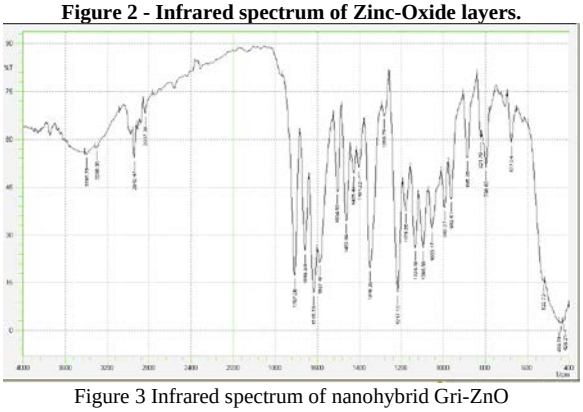


Figure 3 Infrared spectrum of nanohybrid Gri-ZnO

3.2 Diagnosis using X-ray diffraction spectrum

The x-ray diffraction spectrum for nanohybrid Gri-ZnO and ZnO layers was studied to know the difference in the layer thickness before and after the hybridisation of griseovulvin and ZnO.

Figure 4 shows theZnOx-ray diffraction spectrum of level 100 at 31.29^{o} angle for crystal distance (d) equal 0.281 nm and the level 002 at the 34.82^{o} angel for distance equal to 0.259nm. While the level 101 at 36.29^{o} for crystal distance equal to 0.247 nm [16]. Also the ion exchange between the ZnO and the antifungal griseovulvin were done and the results shown five diffractions at the levels 204, 212, 111,102and 101 of Griseovulvin in addition to ZnO diffraction levels. These results confirmed that ZnO still kept its natural state and the antifungal griseovulvin is encapsulated between the ZnO layers as shown in Figure 5.

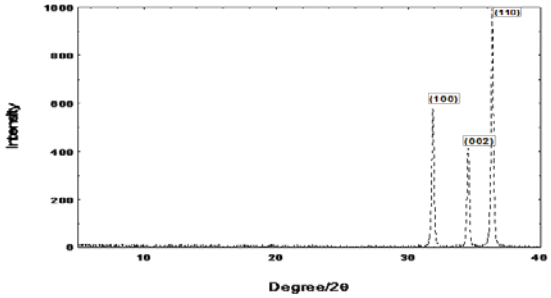


Figure 4 ZnO X-ray diffraction (XRD) spectrum

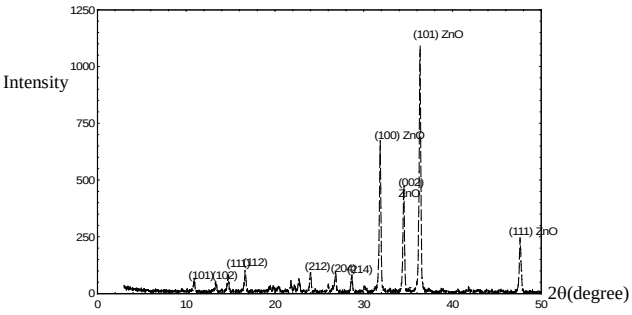
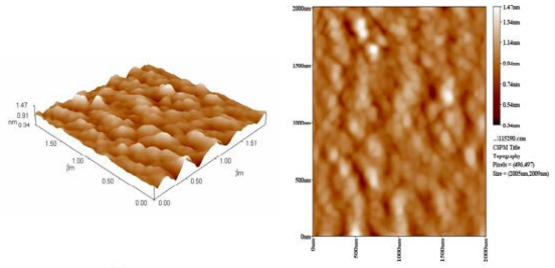


Figure 5 Nanohybrid Gri-ZnOx-ray diffraction (XRD) spectrum



b **a**

Figure 6: A two-dimensional (a) and three-dimensional (b) image of the Gri-ZnO antifungal by atomic force microscope

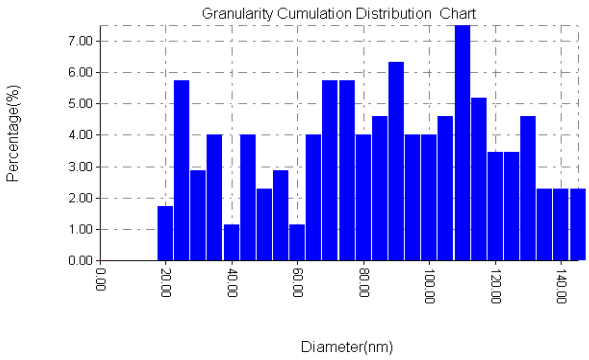


Figure 6 accumulation distributions of nanohybridGri-ZnO molecules after AFM test

3.3 Atomic force microscopy (AFM) screening

The surface morphology of the nanohybrid Gri-ZnO was studied. Figure 6a shows two dimensional photos of the molecular clusters of semi-spherical shapes of antifungal Gri-ZnO. While Figure 6b shows three dimensional photos of a section of surface of the nanohybrid Gri-ZnO. This shows molecular grouping increases with limits of 1.47 nm. This result confirmedthe presence of a new product, the nanohybrid antifungal from the free griseovulvin and ZnO layers.

The molecular size range of nonohybrid antifungal Gri-ZnO is almost 81.85 nm. Preparation of the nanohybrid antifungal gave molecules with diameter ranging from 20 – 100nm (20, 25, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100nm) with corresponding percentages (1.72, 5.75, 2.87, 4.02, 1.15, 4.02, 2.30, 2.87, 1.15, 5.75, 5.75, 4.60, 6.32, 4.02, 4.02% respectively). The crystallity index of the nanohybrid antifungal was found to be 1.75 according to data in Table 1 and XRD spectrum (Figure 5).The current study agreed with another study [15], where the prepared spherical shape of the nanohybrid molecules size (50-76nm) is similar.

Table 1 Diameter, size and aggregation (grouping) nanohybrid antifungal Gri-ZnO molecules after AFM tested

Avg. Diameter:81.85 nm

Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)
20.00	1.72	1.72	65.00	4.02	29.89	110.0	7.47	76.44
25.00	5.75	7.47	70.00	5.75	35.63	115.00	5.17	81.61
30.00	2.87	10.34	75.00	5.75	41.38	120.00	3.45	85.06
35.00	4.02	14.37	80.00	4.02	45.40	125.00	3.45	88.51
40.00	1.15	15.52	85.00	4.60	50.00	130.00	4.60	93.10
45.00	4.02	19.54	90.00	6.32	56.32	135.00	2.30	95.40
50.00	2.30	21.84	95.00	4.02	60.34	140.00	2.30	97.70
55.00	2.87	24.71	100.00	4.02	64.37	145.00	2.30	100.00
60.00	1.15	25.86	105.00	4.60	68.97			

Table 2 The disruption of fungal isolates according to the diseases and body parts' infection

Isolate No	Disease	Fungal name	Body part infected	Patients' age
1	Tinea corporis	Trichophyton mentagrophytes	body	37
2	Tinea faciei	Trichophyton mentagrophytes	Face	25
3	Tinea manuum	Trichophyton mentagrophytes	hand	13
4	Tinea capitis	Trichophyton mentagrophytes	head	5
5	Tinea corporis	Trichophyton mentagrophytes	body	20
6	Tinea corporis	Trichophyton mentagrophytes	leg	16
7	Tinea capitis	Microsporumcanis	Head hair	6
8	Tinea corporis	Microsporumcanis	body	4
9	Tinea corporis	Microsporumcanis	hand	37
10	Tinea cruris	Epidermophytonfloccosum	grion	29
11	Tinea unguium	Epidermophytonfloccosum	Nails	35
12	Tinea pedis	Epidermophytonfloccosum	Athlete's foot	32

Table 3 inhibition activity of the Gri antifungal and nanoyhbrid Cri-ZnO compound against 12 Dermatophytes spp.

Componds average	12	11	10	9	8	7	6	5	4	3	2	1	Fungal isolates	Compond
	Inhibition zone /mm												Comcentration mg / ml	
4.675 B	0	0	0	0	0	0	0	0	0	0	0	0	0.01	Cri -ZnO
	0	0	0	0	0	0	0	0	0	0	0	0	0.1	
	15	0	7.3	6	0	0	6	0	0	0	0	0	0.25	
	15.3	6.3	17.3	3	0	2.6	9.6	5.6	0	0	0	0	0.5	
	17	10.3	24.3	7.3	0	10.3	27	11.3	0	0	0	0	1	
	17.3	11.3	26	7	0	17	29.6	14	0	0	11.6	8	5	
16.138 A	0	0	0	0	0	0	0	0	0	0	0	0	0.01	Cri- free
	0	0	0	0	0	0	0	0	0	0	0	0	0.1	
	0	0	0	0	0	0	0	0	0	0	0	0	0.25	
	35.3	33.3	42.6	30.6	31.3	29.3	35.3	39.3	0	36.6	30	40.6	0.5	
	38.3	38.6	27.3	31	38.3	36	34.6	36.6	0	34.6	43	33.3	1	
	38.6	36.3	30	41.3	34.3	30	37.6	38	0	35.3	30.6	32.3	5	
	14.47 a	11.08 bc	14.58 a	10.52 bcd	8.66 d	10.44 bcd	15.00 a	12.08 b	0.0 e	8.88 d	9.61 cd	9.52 cd	Isolates average	

* The vertically different capitals indicate significant differences ($p < 0.05$) between the compounds.

* The different small letters horizontally indicate significant differences ($p < 0.05$) between fungal isolates.

5	1	0.5	0.25	0.1	0.01	Concentration
21.66 A	20.81 A	18.52 B	1.43 C	0 D	0 D	average

* The different capitals clearly indicate significant differences ($p < 0.05$) between the concentrations used

Interference	Concentration	Isolates	Compound	Factor
7.084	1.446	2.045	0.834	$LSD_{0.05}$

4.3 Inhibition activity of nanohybrid antifungal Gri-ZnO against dermatophyte isolates

The inhibition activity against 12 dermatophyte isolates (numerated from 1 to 12) wasstudied*in vitro*.The dermatophyte isolates included six strains of *Trichophyton mentagrophytes*,which were isolated from three patients suffering from Tinea corporis (isolates No. 1, 5 and 6)and three patients suffering from Tinea faciei, Tinea manuum and Tinea capitis (isolates No. 2, 3 and 4 respectively). Also three strains of *Microsporumcanis*were isolated from patients suffering from Tinea capitis(isolate No. 7) andTinea corporis(isolates No. 8 and 9). Threestrains of *Epidermophytonfloccosum*were isolated from patients suffering from Tinea cruris, Tinea unguium and Tinea pedis(isolates No. 10, 11 and 12 respectively) as shown in Table 2.

The statistical analyses of antimicrobial activity demonstrated that there was a significant differences ($p < 0.05$) between the fungal isolates. The isolates numbered 6, 10 and 12 were the most susceptible to the studied compound, with significant differences between the isolates. Zones of inhibition were 15.00, 14.47 and 14.58 mm respectively. While fungal isolates numbered 1, 2, 3, 8, 9 and 7 were less susceptible to the treatment,no significant differences between the isolates ($P>0.05$) was measured. However, isolate number 4 showed high resistance toward both the free griseovulvin and the hybrid Gri-ZnO as shown in table 3.

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