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Antifungal Activity and Time-Kill Kinetics of Griseofulvin Nanoparticles Generated by Antisolvent Precipitation

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ABSTRACT

Background: Griseofulvin is a fungistatic agent used in the treatment of dermatophytic infections of the scalp, hair and nails of both man and animals.

Objective: This study aimed to assess the antifungal activity and time-kill kinetics of griseofulvin nanoparticles generated by antisolvent precipitation.

Methods: The activities of griseofulvin powder and its nanoparticles generated by antisolvent precipitation against three Dermatophytes - *Trichophyton rubrum*, *Trichophyton tonsurans* and *Epidermophyton floccosum* were determined using standard microbiological techniques. The time-kill patterns of the two forms of the drug were also studied.

Results: The griseofulvin nanoparticles produced wider zones of inhibitions against the test organisms when compared with the pure griseofulvin powder, and the susceptibility of the test organisms to the nanoparticles was found to be in the order: *Trichophyton tonsurans* > *Trichophyton rubrum* > *Epidermophyton floccosum*. Minimum inhibitory concentration values of 1.56 mg/mL (*Trichophyton tonsurans*), 6.25 mg/mL (*Trichophyton rubrum*) and 1.56 mg/mL (*Epidermophyton floccosum*) were obtained for both the griseofulvin powder and its nanoparticles. The time-kill studies showed similar reduction in viable cell count for both the griseofulvin powder and its nanoparticles.

Conclusion: The test organisms are slightly more susceptible to the nanoparticles than the unprocessed drug, and the nanoparticles exhibit faster onset of kill. However, the extents of antifungal action of the two forms of the drug are similar.

Keywords: Griseofulvin, nanoparticles, time-kill kinetics, antifungal activity.

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Activité antifongique et cinétique antifongique des nanoparticules de griséofulvine générées par précipitation antisolvant

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RÉSUMÉ

Contexte : La griséofulvine est un agent fongistatique utilisé dans le traitement des infections dermatophytiques du cuir chevelu, des cheveux et des ongles chez l'homme et l'animal.

Objectif : Cette étude visait à évaluer l'activité antifongique et la cinétique de destruction des nanoparticules de griséofulvine générées par précipitation antisolvant.

Méthodes : Les activités de la poudre de griséofulvine et de ses nanoparticules, obtenues par précipitation antisolvant, contre trois dermatophytes - *Trichophyton rubrum, Trichophyton tonsurans* et *Epidermophyton floccosum* - ont été déterminées à l'aide de techniques microbiologiques standard. Les courbes de fongicidie en fonction du temps des deux formes du médicament ont également été étudiées.

Résultats : Les nanoparticules de griséofulvine ont induit des zones d'inhibition plus étendues contre les organismes testés par rapport à la poudre de griséofulvine pure. La sensibilité des organismes testés aux nanoparticules a été classée dans l'ordre suivant : *Trichophyton tonsurans* > *Trichophyton rubrum* > *Epidermophyton floccosum*. Les concentrations minimales inhibitrices (CMI) obtenues étaient de 1,56 mg/mL (*Trichophyton tonsurans*), 6,25 mg/mL (*Trichophyton rubrum*) et 1,56 mg/mL (*Epidermophyton floccosum*) pour la poudre de griséofulvine et ses nanoparticules. Les études de cinétique antifongique en fonction du temps ont montré une réduction similaire du nombre de cellules viables pour la poudre de griséofulvine et ses nanoparticules.

Conclusion : Les organismes testés sont légèrement plus sensibles aux nanoparticules qu'au médicament non transformé, et les nanoparticules se caractérisent par un début d'action fongicide plus rapide. Cependant, l'ampleur de l'action antifongique des deux formes du médicament est similaire.

Mots-clés : Griséofulvine, nanoparticules, cinétique antifongique en fonction du temps, activité antifongique.

INTRODUCTION

Griseofulvin ($C_{17}H_{17}ClO_6$) is a naturally occurring compound first identified and extracted from Penicillium griseofulvum in 1939. Besides Penicillium, it can be derived from other ascomycete fungi such as *Xylaria flabelliformis*, *Abieticola koreana*, and *Stachybotrys levispora*.[1] It was initially introduced for treating ringworm infections caused by Microsporum canis in guinea pigs.[2] Later studies confirmed its antifungal efficacy against other dermatophytes, including Trichophyton and Epidermophyton species.[3] Approved by the FDA in 1959, griseofulvin is effective for the management of dermatophyte infections in both humans and animals.

Griseofulvin acts by inhibiting the assembly of microtubules, disrupting their function in dermatophytes. This disruption affects the formation of the mitotic spindle, ultimately halting cell division (mitosis). It may also interfere with the synthesis and polymerization of nucleic acids. The drug is actively transported into susceptible dermatophytes through an energy-dependent process, and resistance can develop if this transport mechanism is reduced.[1]

Based on the mode of action, griseofulvin is a fungistatic agent, effectively suppressing the growth of fungi such as *Trichophyton*, *Microsporum*, and *Epidermophyton* species. However, it just is not effective against dimorphic fungi, yeasts like *Malassezia* and *Candida*, or infections such as chromomycosis. Due to its rapid elimination from the body, griseofulvin requires long-term administration to maintain therapeutic levels and achieve clinical effectiveness.[4]

Griseofulvin's low water solubility (8.64 mg/L) and high permeability group it into class II of the Biopharmaceutics Classification System and its low dissolution rate in the gastrointestinal tract reduces its bioavailability and clinical efficacy.[5] Various techniques such as: chemical modification, salt formation, amorphization and particle size reduction can be used to improve the aqueous solubility of poorly soluble drugs. Nanoprecipitation is one of the methods for achieving particle size reduction producing particles of nano-dimension.[6] Nanoparticles (NPs) are ultrafine particles ranging from 1 to 100 nm in size. Their reduced size and increased surface area affect their physical and biological activities.[7]

Antisolvent nanoprecipitation can be carried out with or without incorporation of a surfactant during the dissolution stage. Griseofulvin nanoparticles were generated using the two approaches.8 The ordinary nanoprecipitation produced a 22.4 % reduction in particle size while surfactant-mediated nanoprecipitation produced a 55.5 % reduction yielding particles of Z-average 77.81 nm and 44.71 nm respectively. The aqueous solubility of the nanoparticles generated without the inclusion of a surfactant was observed to be 1.72 folds that of the unprocessed drug while that of the nanoparticles generated with the inclusion of the surfactant was found to be 4.14 folds.

This work aims to determine the antifungal activity and the time-kill kinetics of the griseofulvin nanoparticles generated with the inclusion of the surfactant in comparison with the unprocessed drug. From the previous work of Olorunsola *et al*.,8 the nanoparticles were smaller and more soluble compared with the nanoparticles generated without the inclusion of a surfactant and also compared with the unprocessed drug.

MATERIALS AND METHODS

Materials

The materials used include griseofulvin powder (Thosco, Thode and Scobel, Hamburg, Germany), griseofulvin nanoparticles generated with polysorbate 80 inclusion,[8] Dermatophyte Test Medium (DTM) and Sabouraud Dextrose Agar (SDA).

Test Organisms

Collection of test organisms

The test organisms: Trichophyton tonsurans, Trichophyton rubrum and Epidermophyton floccosum were obtained from the National Veterinary Research Institute, Vom, Plateau State.

Confirmation of test organisms

The dermatophyte isolates were confirmed by sub-culturing on Dermatophyte Test Medium (DTM) and incubating at room temperature for 7 days. The resulting fungal colonies were viewed under the microscope with 10 % KOH to observe the characteristic mycelium.

Maintenance of fungal isolates

The fungal isolates were maintained on Sabouraud Dextrose Agar (SDA) and stored in the refrigerator at 4 ^{o}C.

Susceptibility Testing

The antifungal activity of griseofulvin against the dermatophytes was evaluated by the agar well diffusion method as described by Asuquo and Udobi.[9] The fungal

inoculum was prepared by suspending 3 distinct fungal colonies from a 7 days culture on SDA in 5 mL of sterile water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5 x 10^6cfu/mL. A 1 in 10 dilution of standardized suspension was carried out in sterile water to obtain the inoculum size of 5 x 10^5cfu/mL. A 0.1 mL quantity of the final dilution of the test organism was aseptically introduced into the labelled Petri dishes. Twenty millilitres (20 mL) of sterilized Sabouraud dextrose agar medium was aseptically poured into each petri dish and gently swirled to mix. The plates were allowed to set and equidistant wells were created on the surface of the agar using a sterile 4 mm standard cork borer. Different concentrations (0.5, 0.25 and 0.125 mg/mL) of the drug (powder/nanoparticles) diluted in sterile water were introduced into the different wells and labelled appropriately. The plates were incubated at room temperature for 3-7 days after which the diameters of inhibition zones were taken in duplicates.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MIC) of griseofulvin/nanoparticles against test dermatophytes were determined by the broth dilution method as recommended by EUCAST using sterile test tubes.[10] A 2 mL quantity of Sabouraud Dextrose Broth (SDB) prepared according to the manufacturer's instruction was aseptically dispensed into each of 5 sterile test tubes. Each formulation was diluted with sterile water to a concentration of 25 mg/mL. This concentration was then used to carry out a two-fold serial dilution along the 5 sterile test tubes to obtain a concentration between 12.5 mg/mL and 0.78 mg/mL.

The fungal inoculum was prepared by suspending five distinct fungal colonies from a 7 days culture on SDA in 5 mL of sterile water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5 x 10^6cfu/mL. A 1 in 10 dilution of the standardized suspension was carried out in sterile distilled water to obtain an inoculum size of 5 x 10^5 cfu/mL. A 0.1 mL quantity of the fungal suspension was introduced into the different test tubes containing the different concentrations of the nanocrystalline griseofulvin or pure griseofulvin formulation accordingly. The inoculated test tubes were incubated for 3-7 days at room temperature. The lowest concentration of the formulation that showed no visible turbidity after incubation was recorded as the minimum inhibitory concentration (MIC) for each of the test organisms.

Time-Kill Assay

The method for the in vitro time-kill assay of fungi as described by Correa-Royero *et al*.11 and Zerroug *et al*.12 was adapted for this test. Different test tubes containing 3 mL of Sabouraud Dextrose Broth (SDB) were labelled according to the test organisms. A measured 1 mL of the drug dispersion was introduced into a test tube such that a concentration equivalent to the MIC for the organism will would be obtained. A fungal suspension was aseptically transferred to a test tube and diluted to obtain an initial inoculum cell density of approximately 5 × 105 cfu/mL. Thereafter, 1 mL of the standardized inoculum of broth cultures of each fungus was aseptically inoculated into the different test tubes as labelled. The test tubes were incubated at room temperature with shaking. At intervals., 0.1 mL sample was withdrawn from each test tube and diluted to a standard inoculum size before aseptically transferring into sterile petri dishes containing Sabouraud dextrose agar (SDA) medium in quadruplicates. The plates were incubated at room temperature for 72 hours. After the incubation period, the number of fungal colonies on each plate was enumerated by direct counting. The mean colony count was computed over time. The data were used to generate a range of time kill curves for each organism.

RESULTS

Confirmation of Fungal Isolates

Results of the cultural and microscopic characteristics obtained for the test organisms which were used for their identification are presented in Table 1.

Table 1: Cultural and microscopic characteristics of the test isolates

S/N	Organism	Cultural characteristics	Microscopy
1	*Trichophyton tonsurans*	Whitish colonies which are brownish on reverse with raised borders.	Septate hyphae with few irregular macroconidia and more microconidia
2	*Trichophyton rubrum*	Whitish colonies which are brownish on reverse with raised borders.	Septate hyphae and abundant macroconidia by the sides
3	*Epidermophyton floccosum*	Khaki surface, raised centre, brown on reverse.	Thin walled, smooth surface and circular in shape macroconidia

Susceptibility of the Isolates to the Drug

The zones of inhibition of the test organisms due to the activity of griseofulvin and the nanoparticles are presented in Tables 2. The nanoparticles were observed to be more active than the drug powder showing comparatively wider zones of inhibition. The susceptibility of the test organisms to the nanoparticles is in the order: *Trichophyton tonsurans* > *Trichophyton rubrum* > *Epidermophyton floccosum*.

Table 2: Susceptibility of the fungal isolates to the drugs

S/N	Drug Conc. (mg/mL)	Organism	Zone of inhibition by unprocessed drug (mm)	Zone of inhibiti on by nanoparticles (mm)
1	50	*Trichophyton tonsurans*	20.00	25.00
		Trichophyton rubrum	20.00	20.00
		Epidermophyton floccosum	10.00	20.00
2	25	*Trichophyton tonsurans*	15.00	20.00
		Trichophyton rubrum	18.00	18.00
		Epidermophyton floccosum	8.00	15.00
3	12.5	*Trichophyton tonsurans*	13.00	18.00
		Trichophyton rubrum	15.00	15.00
		Epidermophyton floccosum	-	12.00

Minimum Inhibitory Concentration (MIC)

Effects of the drug powder and the nanoparticles concentrations on the fungal isolates are shown in Table 3. The minimum inhibitory concentration in each case is represented by the lowest drug concentration at which no growth was observed. The MIC of the nanoparticles against a particular organism is not different from that of the unprocessed drug. Both forms of the drug had MIC values of 1.56 mg/mL for *Trichophyton tonsurans*; 6.25 mg/mL for *Trichophyton rubrum* and 1.56 mg/mL for *Epidermophyton floccosum*.

Table 3. Effect of drug concentration on fungal growth

S/N	Drug	Organism	Concentration (mg/mL)				
			12.5	6.25	3.125	1.56	0.78
1	Unprocessed	*Trichophyton tonsurans*	-	-	-	-	+
		Trichophyton rubrum	-	-	+	+	+
		Epidermophyton floccosum	-	-	-	-	+
2	Nanoparticles	*Trichophyton tonsurans*	_	_	_	_	+
		Trichophyton rubrum	_	_	+	+	+
		Epidermophyton floccosum	_	_	_	_	+

Key: + = Growth: - = No growth

Time Kill Kinetics

Plots of mean colony counts against time, showing the kill kinetics of griseofulvin powder and the nanoparticles against *Trichophyton tonsurans, Trichophyton rubrum* and *Epidermophyton floccosum* are shown in Figures 1a, 1b and 1c respectively. The nanoparticles, and the pure powder show an appreciable reduction in colony forming units against *Trichophyton tonsurans, Trichophyton rubrum* and *Epidermophyton floccosum* respectively.

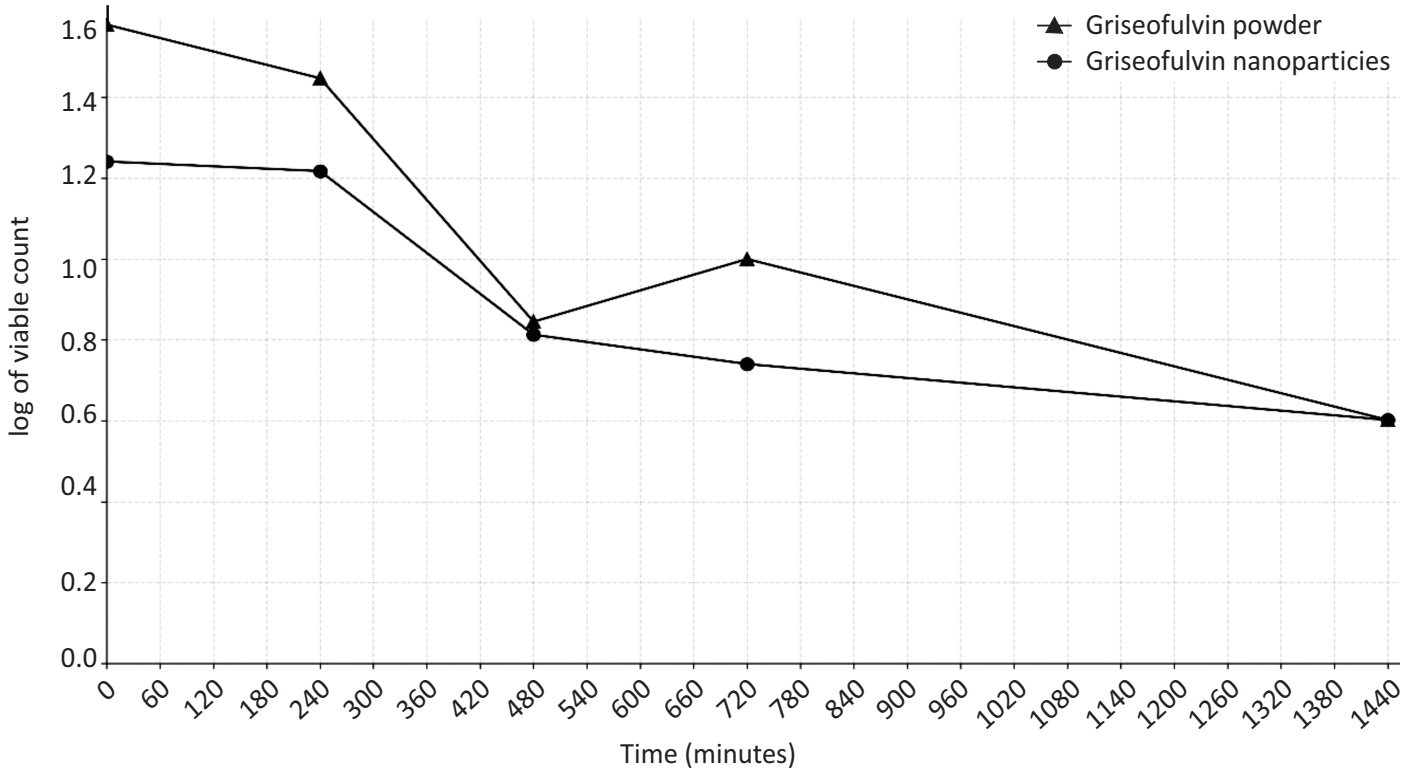


Figure 1a: Time-kill curve of griseofulvin powder and the nanoparticles against *Trichophyton tonsurans* (▲ = Griseofulvin powder; ● = Griseofulvin nanoparticles)

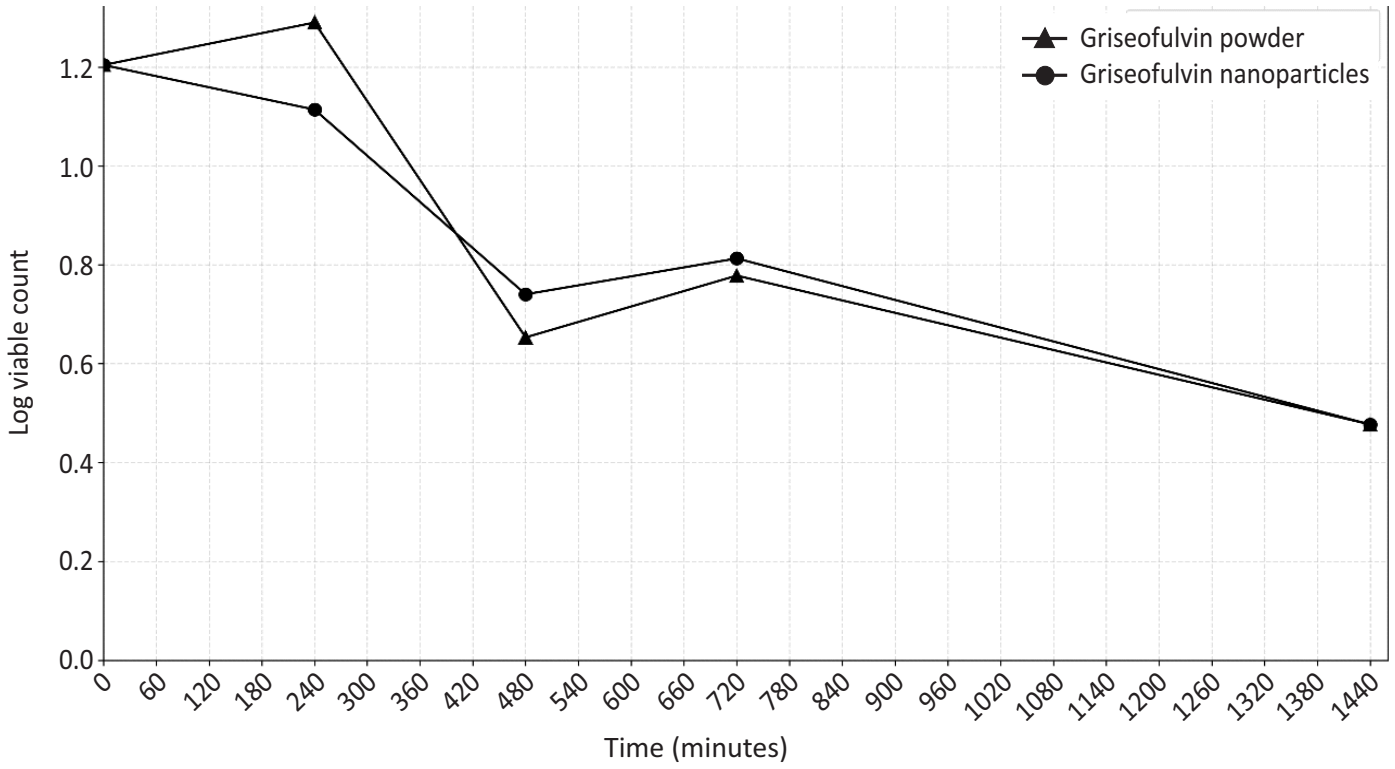


Figure 1b: Time-kill curve of griseofulvin powder and the nanoparticles against *Trichophyton rubrum* (▲ = Griseofulvin powder; ● = Griseofulvin nanoparticles)

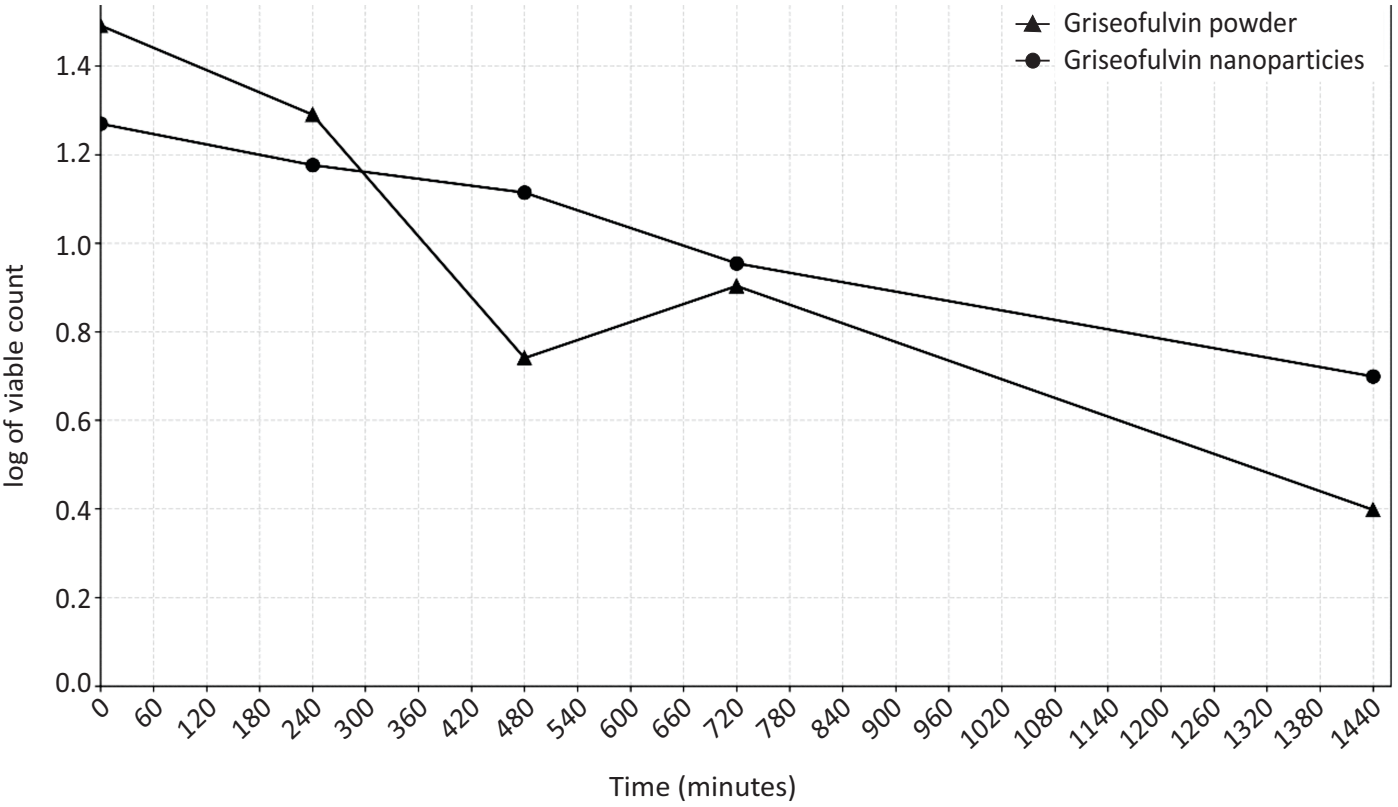


Figure 1c: Time-kill curve of griseofulvin powder and the nanoparticles against *Epidermophyton floccosum* (▲ = Griseofulvin powder; ● = Griseofulvin nanoparticles)

DISCUSSION

Fungal Isolates

Trichophyton tonsurans, Trichophyton rubrum and *Epidermophyton floccosum*, the test dermatophytes which were used for this study are known to be associated with dermatophytosis. Other fungi that are implicated in dermatophytosis are those in the genera *Microsporum, Arthroderma, Nannizzia, Paraphyton* and *Lophophyton*.[13] The World Health Organization estimates that dermatophytosis impacts 20 to 25 percent of the world's population.[14] Infections due to dermatophytes which affect the keratinized tissues of man and animals are of serious concern worldwide. This may be due to the fact that there is a high rate of reinfections after treatment reported among other complaints. The need for improvement in the agents used in the treatment continues to arise.

Susceptibility

Although there are agents for the treatment of dermatophytosis, the development of fungal drug resistance, drug-related toxicity, drug interactions, insufficient bioavailability, immunosuppression due to prolonged use, and non-compliance to conventional antifungal drugs, have encouraged the search for better alternatives. The griseofulvin nanoparticles are characterized by greater capacity to inhibit the investigated dermatophytes as shown by the zones of inhibition. At the three concentrations used (0.50, 0.25 and 0.125 mg/mL), Trichophyton tonsurans and Epidermophyton floccosum were more susceptible to the nanoparticles than the unprocessed drug.

Previous work of Olorunsola *et al*.[8] showed that the nanoparticles of griseofulvin are characterized by better aqueous solubility compared to the unprocessed drug. Therefore, the wider zone of inhibition observed with the nanoparticles may be related to the improved solubility and better distribution.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the powder and the nanoparticles are identical for each organism. Trichophyton rubrum was characterized by the highest minimum inhibitory concentration of the two forms of the drug. Hence, the organism requires more of the drug to be inhibited.

The minimum inhibitory concentration (MIC) of an agent is the lowest concentration of that substance that inhibits the visible growth of the microorganism (fungus) after the incubation period. It is a key tool in evaluating the antimicrobial efficacy of compounds.[15] In this study, the activity of the nanoparticles and that of the unprocessed drug are similar for the organisms because the chemical nature of the two substances is the same being griseofulvin.

Time-Kill Kinetics

The treatments were carried out at the minimum inhibitory concentration for each organism. Both the powder and the nanoparticles resulted in a reduction of colony counts of the test organisms over time. The initial lower count of test organisms at the onset of the kill when exposed to the nanoparticles can be attributed to the higher solubility due to the smaller particle size of the nanoparticles as reported in the previous work.[8] This implies faster onset of antifungal action of the nanoparticles compared to the powder.

The counts after 24-hour exposure to the powder and the nanoparticles were similar for the same organism species showing that the extent of kill was similar. This is further confirmed by the growth observed between time 0 h and 4 hrs in the case of Trichophyton rubrum showing the low activity of the unprocessed drug against the organism during that period when compared with the nanoparticles. Variability in susceptibility and growth patterns is common in dermatophytes and may be related to differences in species.[3]

A similar pattern was observed with *Trichophyton tonsurans* and *Epidermophyton floccosum*. The two organisms were of lower counts at the beginning of the kill on exposure to the nanoparticles. However, the counts at Time 24 hour were similar for the two forms of the drug. The initial low count of fungi for the preparations exposed to the nanoparticles can also be attributed to the fast drug release associated with griseofulvin nanoparticles as shown in a previous work.[16]

CONCLUSION

Nanoparticles of griseofulvin, just like the unprocessed powder, showed good inhibitory effect against dermatophytes with no difference in the minimum inhibitory concentrations of the two forms of the drug against each test organism. The susceptibility of the test organisms to the nanoparticles is higher than that of the unprocessed drug. Furthermore, the nanoparticles are characterized by faster onset of kill. However, the extents of antifungal action of the two forms of the drug are not different.

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