ANTIFUNGAL ACTIVITY OF ZINC OXIDE NANOPARTICLES AGAINST DERMATOPHYTIC LESIONS OF CATTLE

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Abstract. Ringworm is a fungal and zoonotic infectious disease, caused by different species of dermatophytes. In this study, skin scrapings and hair samples were collected from 50 cattle with clinical symptoms of dermatophytosis. The collected samples were directly examined for fungal elements by direct microscopy. Isolates of Dermatophytes were found to be 14.3% and 26.6% for samples obtained from cow and buffaloes respectively. The distribution of isolates was *Trichophyton mentagrophyte* (33.33%), *Micsporum canis* (26.67%), *Candida albicans* (26.67%), *Aspergillus fumigatus* (13.33%) respectively. The antifungal activity of zinc oxide (ZnO) nanoparticles was evaluated for *Trichophyton mentagrophyte*, *Microsporum canis*, *Candida albicans* and *Aspergillus fumigatus*. The largest inhibition in the germination of all the tested fungi was observed at largest ZnO nanoparticles concentration (40 mg/mL).

Key words: ZnO nanoparticles, hexagonal, antifungal activity, *Trichophyton mentagrophytes*, *Microsporum canis*, *Candida albicans*, *Aspergillus fumigatus*.

INTRODUCTION

Ringworm is a fungal and zoonotic infectious disease caused by different species of dermatophytes. These microorganisms are a group of closely related fungi which utilize keratin and tend to be confined to the superficial integument, including skin, nails, claws and hair of both animals and humans [5, 1]. Dermatophytes can be divided into three groups: anthropophilic, zoophilic and geophilic species, depending on their natural habits and host preferences. Zoophilic species of dermatophytes including *Microsporium canis*, *Trichophyton verrucosum*, and *Trichophyton mentagrophytes are* associated with dermatophytosis in wild and domestic animals [10, 25].

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Dermatophytosis can be transmitted from animal to animal and from human to animals. Zoophilic fungi prefer animals as hosts but often cause acute inflammatory reactions when they invade humans [35]. Cattle ringworm mainly occurs in young animals (calves) and is rapidly spread in the herd via infected propagules (hyphae and specialized fungal spores named arthrospores).The disease is responsible for great economic losses due to skin injuries and many casualties in animal products (wool, meat, etc.) [39, 41].

Trichophyton verrucosum is the most common etiologic agent of cattle. *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton simii* and *Microsporum gypseum* are also bovine caused dermatophytosis [17, 28]. The emerging infectious diseases and the development of drug resistant pathogenic bacteria and fungi at an alarming rate is a matter of serious concern. Despite the increased knowledge of microbial pathogenesis and application of modern therapeutics, the morbidity and mortality associated with the microbial infections still remain high [18]. In recent times, the advances in the field of nanoscience and nanotechnology have brought to fore the nanosized inorganic and organic particles which are finding increasing applications as amendments in industrial, medicine and therapeutics, synthetic textiles and food packaging products [15]. Nanoparticles usually ranging in dimension from 1–100 nanometres (nm) have properties unique from their bulk equivalent. With the decrease in the dimensions of the materials to the atomic level, their properties change [24]. The nanoparticles possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired applications [13].

Nanotechnology has the potential to revolutionize the agriculture and food industry too with new tools for molecular treatment of diseases, rapid disease detection, enhancing the ability of plants to absorb nutrients [26]. The antimycotic effects of ZnO and MgO nanoparticles having average size of ~30±10 nm and ~50 ±10 nm respectively are tested on some pathogenic fungi [40]. R.V. Ravishankar *et al*. [30] mentioned that zinc oxide (ZnO) and copper oxide nanomaterials due to their antimicrobial property are being incorporated into a variety of medical and skin coatings. ZnO nanoparticles are used in the wallpapers in hospitals as antimicrobials. ZnO powder is an active ingredient for dermatological applications in creams, lotions and ointments on account of its antibacterial properties [21].

The present study was designed to conduct laboratory diagnosis cutaneous fungal infections among cattle showing typical diagnostic features of superficial and cutaneous mycoses and to evaluate the *in vitro* inhibitory effect of zinc oxide nanoparticles (ZnO) on the growth of dermatophyte isolates.

MATERIALS AND METHODS

In the present study, ZnO nanoparticles (ZnONPs) were prepared using Pechini method [29]. In this technique, stoichiometric amounts of Zn $(NO_3).6H_2O$ were weighed and well mixed, Fig. (1), with an equimolar solution of citric acid:

ethylene glycol. This mixture was then transferred on a magnetic stirrer with a hot plate until drying. After that, an autoignition takes places resulting in a fluffy white yellowish powder. This powder was collected and then heated at 500 ^{o}C in Lenton Furnace UAF 16/5.

The X-ray powder diffraction (XRD) was carried out using a Proker D8 advance X-ray diffractometer with CuK_{α} radiation (λ = 1.5418 Å) for the samples in the range of 20–80°. The crystalline phases were identified using the International Centre for Diffraction Data (ICDD) card. The shape and morphology of the nanoparticles were analyzed using a High Resolution transmission electron microscope (HRTEM) model (JEOl-2100).

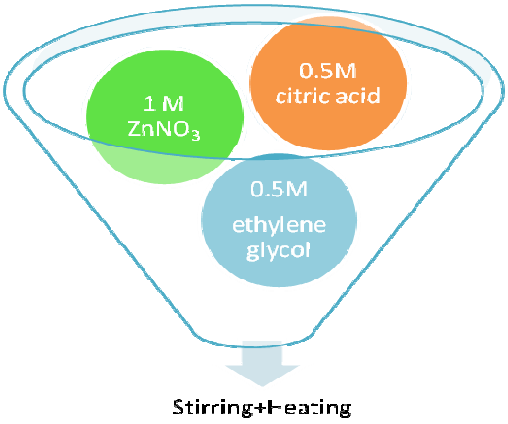


Fig. 1. Flow chart of preparation.

SAMPLES COLLECTION

A total of fifty samples of skin scraping and hair were collected from the site of lesion after cleaning by cotton soaked in 70% alcohol from infected buffaloes and cows which have clinical manifestations of dermatophytosis. Direct microscopical examination used (KOH 20%).

DIRECT MICROSCOPICAL EXAMINATION USING (KOH 20%)

One of two drops of 20% KOH (potassium hydroxide) were placed on a microscopic slide and a small amount of the specimen was added and then, the slide was gently passed through a low flame and covered by a cover slip. After 2 h, the specimen was examined for the presence of arthrospores and hyphae under a light microscope [12].

ISOLATION AND IDENTIFICATION OF DERMATOPHYTES FROM SAMPLES

Scrapings of skin and hair were reduced in size to pieces approximately 1 mm across and the hair roots were cut into similarly sized fragments. Each sample was cultured on the surface of Sabourad Dextrose Agar (SDA) containing chloramphenicol and cyclohexamid and without cyclohexamid to isolate nondermatophytes. The culture media were incubated at 25 °C and 30 °C for up to 5 and 21 days. After isolation the cultures were transferred to freshly prepared SDA media to obtain pure cultures. Pure cultures were also maintained in SDA slants at 5±1 °C. The test dermatophytes were identified by their cultural morphology and microscopic characteristics [8, 35].

IDENTIFICATION OF DERMATOPHYTES

The identification was based on colonial appearance, pigment production and the micro morphology of the spores produced. Cultures were examined at 4 or 5 days intervals from the onset. Some characteristics were also noted on the texture, colour and shape of the upper thallus and the production of pigment on the underside [9, 12, 33].

IN VITRO ANTI-FUNGAL ASSAYS

The following procedure was used according to [20]. Fungal spores were harvested after 7 days old on SDA slant. Culture was washed with 10 mL normal saline in 2% Tween80 with the aid of glass beads to help in the dispersion of the spores. The spore suspensions were standardized to 105 spores/mL. SDA was prepared according to specifications, autoclaved at (121°C for 15 minutes) and supplemented with 0.05%chloramphenicol and dispensed into 11 cm diameter Petri dishes. One mL of each standardized spore suspension (10^5 spores/mL) was evenly spread on the surface of the SDA plates. Then, by using a special instrument known as sterile cork borer (6 mm in diameter), wells were made on the surface of SDA plates. The ZnO nanoparticles were taken at different concentrations of 5, 10, 20, 30 and 40 mg/mL, each concentration dissolved in sterilized distilled water using an ultrasound bath. Each well was inoculated with 100 μL from each concentration used. Further the plates were incubated at 30 °C for 5 days and the growth inhibition was examined by the formation of inhibitory zone. The diameters of the inhibition zones were measured in millimetres (mm).

RESULTS AND DISCUSSION

X-ray diffraction pattern (Fig. 2) of the ZnO nanopowder reveals single phase with no extra peaks indicating the high purity of the sample under investigation. The data were compared and indexed with ICDD card no 89-1397. The sample

crystallized in hexagonal structure with space group P63mc. The crystallite size was calculated from Debye's sheerer formula using the full width at half maximum of the most intense peak. The results reveal that an average crystallite size of 57 nm is obtained.

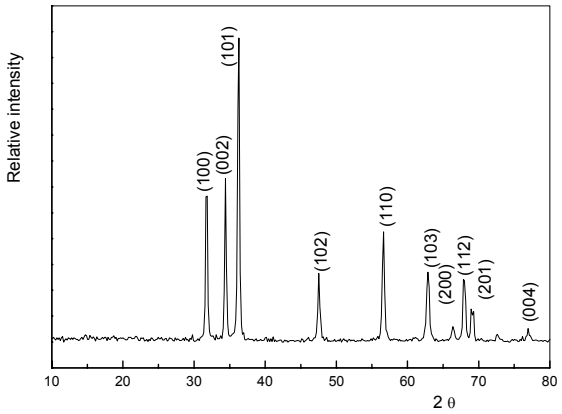


Fig. 2. X-ray diffraction pattern of ZnO particles after annealing at 500 ^{o}C.

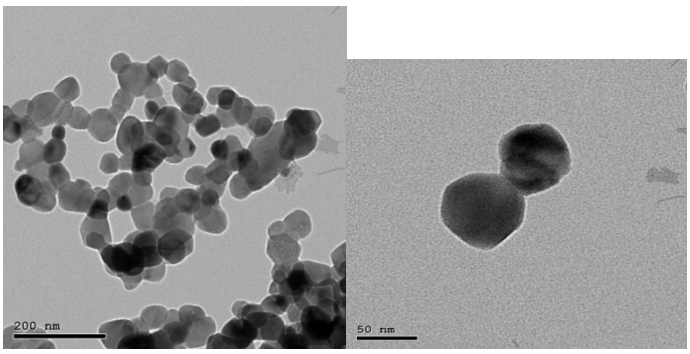


Fig. 3. High resolution transmission electron micrograph of ZnO nanoparticles.

High resolution transmission electron micrograph shows clear platelets with hexagonal shape, slightly agglomerated in a chain like network. Homogeneous size and distribution with average particle size 60 nm. This result agrees well with that calculated from XRD.

Dermatophytes are the most common agents of fungal infections worldwide [42]. Dermatophytic infections have been considered to be a major public health problem in many parts of the world. The infections are common in the developing countries, and are of particular concern in the tropical ones. Microsporum and Trichophyton species are the most common pathogens in skin infections. Less frequently, superficial skin infections are caused by nondermatophytes fungi (e.g., *Candida* species [32].

Table 1

Clinical diagnostic features of cattle ringworm with special reference to direct examination, positive microscopical sample with KOH (20%) and culture results

Animal species	No. of animals	No. of positive cases (clinical signs)		Positive microscopically sample with KOH (20%)		Positive culture for dermatophytes	
		No.	%	No.	%	No.	%
Buffaloes	15	11	73.3	7	46.7	4	26.6
Cow	35	30	85.7	10	28.6	5	14.3

Direct examination of the skin scrapings using 20% KOH revealed fungal mycelia and arthrospores in the macerated debris and infected hairs, characterized by large-spore ectothrix arrangement of arthrospores. Mycelia were seen within the hair shaft running parallel to the length of the hair. A direct Gram's stain was carried out on smears of the scrapings, revealing dense forms of Gram-positive branching filaments. As demonstrated in Table 1 out of a total number of 50 buffaloes and Cow, 41 cases showed clinical signs of ringworm suspected to be dermatophytosis on visual inspection (circumscribed, circular, greyish and crusty lesions).The results of direct microscopic examination with KOH (20%) showed that 46.7% of samples obtained from buffaloes and 28.6% of those obtained from cows had positive results in direct examinations. Not all of the KOH positive samples were culture positive on SDA, only 4 (26.6%) from buffaloes and 5 (14.3%) from cows. These results come in agreement with those obtained [3, 11, 16] who showed that 15.2% of cattle in age group of less than 2 years and 9% of those in age group of more than 2 years were positive for dermatophytosis.

The obtained results in Table 2 showed that the most common dermatophytes isolated was *Trichophyton mentagrophyte*, 5 strains (33.33%), and the most common non dermatophytes isolated was *Candida albicans* strains (26.67%). Cabanes and his colleagues recorded that the most frequent dermatophytes isolated in domestic animals were *Microsporum canis* (55.9%), *Trichophyton mentagrophyte* (27.2%), *Microsporum gypseum* (7.4%) and *Trichophyton verrucosum* (7.4%) [7]. Al-Ani *et al*. [4] reported that *T. verrucosum* was the most commonly identified (47.88% of the total fungi isolated). *T. mentagrophytes* was the second frequent isolated fungi from calves with ringworm (12.68% of the total isolated fungi). Other fungi isolated with low frequency included *Trichophyton schoenleinii*, *Trichophyton terrester*, *Trichophyton violaceum*, *Microsporum nanum*, *Microsporum distortum*, *Microsporum audouinii*, *Alternaria* species, *Fusarium* species, *Penicillium* species, *Cephalosporidium* species and *Aspergillus flavus* and *Aspergillus fumigates*. *Trichophyton verrucosum* was the fungal [36] species responsible for cattle dermatophytosis and J.M. Sudd *et al*. [38] mentioned that four species of dermatophytes were identified out of 35 positive cultures for cows. These species were *Trichophyton rubrum* (19 isolates), *Trichophyton*

verrucosum (10 isolates), *Trichophyton mentagrophytes* (5 isolates) and *Microsporum canis* (one isolate).

The skin of animals is contaminated by numerous fungi, some of which are opportunistic pathogens or allergens. The length of time that fungi can survive on the skin of animals and whether some fungi can multiply on the coats of animals contaminated by soil and plant material are unknown. The presence of opportunistic fungi on the coats of animals creates an opportunity for them under special circumstances to become invasive of the skin or hair and thus cause primary or secondary infections [31].

Microsporum canis, *Trichophyton verrucosum* and *Trichophyton mentagrophytes* are common agents of ringworm in animals, but they are also frequently associated with human infection which results from a direct contact with cattle or infected fomites [2]. The amount of literature on human infections due to the three dermatophytes is enough evidence of their human affinity. Of the three, *Microsporum canis* is the best documented. This is mainly because it causes a lot of scalp ringworm in children. *Microsporum canis* commonly infects pet animals and especially cats and dogs which shed infective particles into the domestic environment and contact with these results in familial infections [6]. Like other types of ringworm, young children particularly in the age range 5–14 years are more susceptible to infection than adults. Similarly, kittens and puppies are more susceptible to ringworm than adult animals. *Microsporum canis* is also known to cause ringworm in horses, monkeys, apes and chinchillas [14]. *Trichophyton mentagrophytes* var. interdigitale, a member of the *Trichophyton mentagrophytes* complex, is essentially a cause of tinea pedis and tinea cruris, and does not invade hair *in vivo* [23].

A.C. Pier *et al*. [27] reported that *Trichophyton mentagrophyte* has been usually associated with toe nail onychomycosis. Dermatophytes were the most common group followed by *Candida albicans* and non-dermatophyte *Aspergillus* in the aetiology of onychomycosis [22].

The results of the quantitative antifungal assessment by well diffusion method are reported in Table 3 and Figure (4 A–C) from which it is observed that the size of the inhibition zone is larger for all isolated dermatophytes and nondermatophytes at concentration of 40 mg/mL ZnO nanoparticles than the 5 ,10, 20 and 30 mg/mL. The reason for the difference in the antifungal activity for different test microorganisms may be due to the difference in structure and thickness of the cell wall membrane. The antifungal activity was found to depend strongly on the concentration. The presence of high inhibition zone with nearly no growth occurrence against tested strains clearly indicate that the mechanism of the fungicidal action of ZnO involves disrupting the membrane. These results agree with those obtained [19, 37] who recorded the ability of ZnO nanoparticles to

affect the viability of the pathogenic yeast, *Candida albicans*, as well as a concentration-dependent effect. The minimal fungicidal concentration of ZnO was found to be 0.1 mg/mL. This concentration caused an inhibition of over 95% in the growth of *C. albicans*. ZnO nanoparticles also inhibited the growth of *C. albicans* when it was added at the logarithmic phase of growth.

Table 2

Identification of dermatophytes and nondermatophytes isolated from infected animals

Fungal isolates	Number of isolates (n = 15)	Percentage%
Dermatophytes		
Trichophyton mentagrophyte	5	33.33
Micsporum canis	4	26.67
Nondermatophytes		
Candida albicans	4	26.67
Aspergillus fumigatus	2	13.33

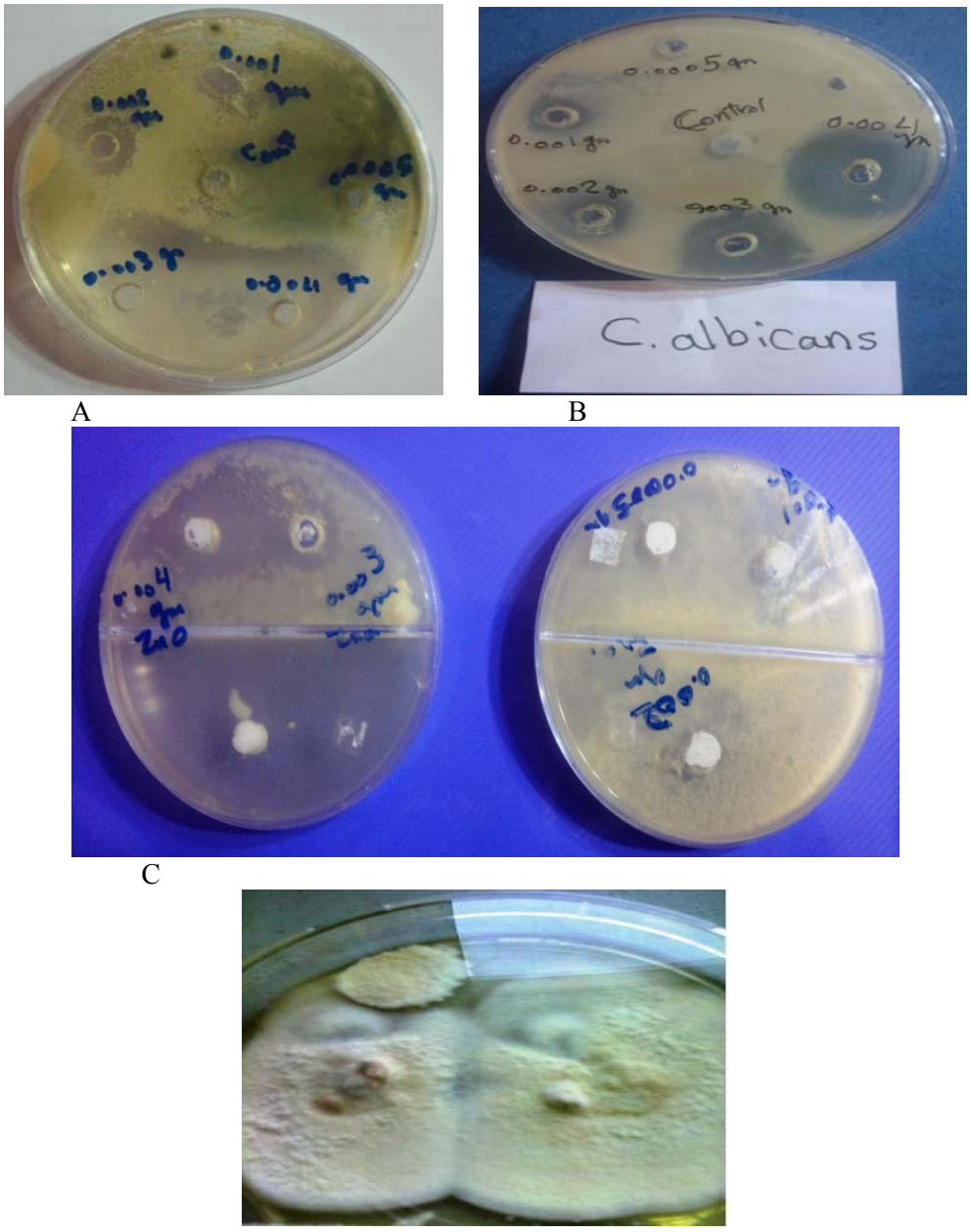
Table 3

Antifungal assessment by well diffusion method

Fungus	Diameter of the clear inhibition zone* (mm)				
	5 mg/mL	10 mg/mL	20 mg/mL	30 mg/mL	40 mg/mL
Trichophyton mentagrophyte	–ve	–ve	2	16	22
Micsporum canis	–ve	–ve	3	17	23
Candida albicans	–ve	4	10	21	30
Aspergillus fumigatus	–ve	4	10	30	38
Control (distal water)	–ve	–ve	–ve	–ve	–ve

*–ve = no inhibition zone.

J. Sawai *et al*. [34] evaluated antifungal activity of metallic oxides (MgO, CaO and ZnO) by an indirect conductimetric assay. Their results indicated that CaO and MgO had antifungal activity above 1600 ppm against *S. cerevisiae* and other fungi. However, ZnO exhibited only a weak antifungal activity against *S. cerevisiae*, but some growth inhibition was observed at 100 ppm.



A B C D

Fig. 4. Antifungal activity of ZnO nanoparticles against tested strains (A. *Aspergillus fumigatus*, B. *Candida albicans*, C. *Trichophyton mentagrophyte*; D. shows *Microsporum canis* on SDA as control.

L.E. Shi *et al*. [37] mentioned that ZnO nanoparticles had fungicidal effect against yeasts and also a fungiostatic action against moulds. The difference between yeasts and moulds could be attributable to the difference in their cell wall and membrane structure. Chitin makes up to 45% of the cell wall of *Aspergillus niger*; however, it is present only 3% in *Saccharomyces cerevisiae*. For almost all fungi, the central core of the cell wall is a branched β-1, 3, 1, 6 glucan that is linked to chitin via a β-1, 4 linkage. The binding of the oxides particles on the fungal cell surface through electrostatic interactions could be a possible mechanism.

CONCLUSIONS

In conclusion, the results of the present study highlight the importance of cattle ring worm as an economically important zoonotic infection. According to findings of this study ZnO nanoparticles showed significant antifungal properties. Consequently, in the field of elimination of ringworm infection, ZnO nanoparticles could be proper candidates disinfectant agents and could be used as active ingredient for dermatological applications.

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